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Lewis antigens are blood group carbohydrate antigens implicated as potential target antigens in breast cancer. Studies supported by this grant are intended to develop reagents that better target these antigens which might prove to be valuable as immunotherapeutics for breast cancer treatment. In the past year, we concentrated on defining recognition properties of anti-Lewis Y antibodies that confer their specificity for Lewis Y. We observed important differences among cancer cells in their mode of interaction with endothelium based on carbohydrate-antibody interactions, which strongly suggest a non-selectin mediated pathway for some breast adenocarcinoma adherence. We expanded our recognition studies to identify peptides on phage display libraries that mimic the Y antigen. We have shown that immunization with such peptides induce anti-carbohydrate immune responses in mice that react selectively with human breast adenocarcinoma cells. We found that this sera and monoclonals are also cross-reactive with carbohydrate structures on HIV, inhibiting cell free HIV infection of target cells in vitro. The development of peptides that mimic carbohydrates represents a novel approach that may lead to a new type of therapeutic agent, as well as an agent that is generally useful.

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Thomas Li-Tsuen 9/5/95

PI - Signature Date

Lewis Y Antigen as a Target for Breast Cancer Therapy

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INTRODUCTION

Lewis antigens are blood group carbohydrate antigens implicated as potential target antigens in a number of cancers (1-4). The expression of $\alpha 1 \rightarrow 2$ fucosylated structures such as Lewis Y (LeY), H-2 and Lewis b (Le^b) is inversely correlated with the survival of patients with primary lung adenocarcinoma, suggesting that these determinants promote invasiveness (5). The LeY determinant is postulated to play a role in adhesion of breast but not colon adenocarcinoma cells to cytokine activated endothelial cells (6,7). Antibodies that recognize H-2, LeY, and Le^b inhibit the motility of a variety of human tumors (7) and inhibit metastasis of mouse tumors containing these determinants (8).

Colon carcinoma cells adhere to activated human umbilical cord endothelial cells (HUVEC) via a selectin-mediated mechanism. Adhesion can be blocked with anti-selectin antibodies, whereas anti-LeY antibodies do not affect this adhesion process for these cells. Adhesion of breast adenocarcinoma cells is not mediated through selectins, as anti-selectin antibody in a preliminary experiment did not affect adhesion (Results section). However, both anti-LeY and anti-sialyl Lewis a (Le^a) antibodies can block adhesion to HUVEC (7). Interestingly, Type 2 lactoseries structures Y and X are present on the same glycoproteins isolated from SkBr5 mammary adenocarcinoma cells as determined using Y specific antibodies (Results section). This suggests that isolated glycoproteins of mammary adenocarcinoma, in addition to mediating cytotoxic function of Mab, are the major carriers of ligands such as X carbohydrate determinants for adhesion molecules, facilitating the metastatic process. It was previously determined that metastatic potential of the tumor cell correlates with the amount of ligands for E-selectin such as X and SA-X associated with lamp-1 glycoprotein; LeY determinant was also found to be carried by lamp-1 in breast carcinoma cells (9,10). Thus, other yet uncharacterized components of complex LeY and X reactive glycoproteins may also be utilized in carbohydrate-selectin or other yet unidentified lectin interaction of tumor cells to endothelial cells.

The basis of the current program is to utilize structural information for LeY-antibody interactions to develop novel immunotherapeutics for breast cancer treatment. It is postulated that the Y determinant on human breast adenocarcinoma cells is of key importance since it mediates internalization and lethal function of Y specific MAb. Molecular probes based on structural information and newly developed MAbs or fragments can be applied for future diagnosis in tumor progression and micrometastasis as well as immunotherapy. In addition, it appears that anti-idiotypic antibodies that mimic the LeY carbohydrate antigen represent potential surrogate immunogens for specific immunization for the treatment of breast cancer. During the current funding period, we have examined the meditation of adhesion of breast adenocarcinoma cells to endothelium using anti-Y antibodies, examined the molecular basis of recognition of anti-Y antibodies for the Y antigen using molecular modeling, have examined how peptides can mimic the Y antigen by screening a peptide library against two anti-Y monoclonals and whether peptides that mimic carbohydrates can induce humoral responses in mice that can target tumor cells. Antibodies available for this study have been previously described to bind to the Y antigen on breast and other carcinoma cells (1,11,12). The monoclonal BR55-2 (IgG3) and its isotype switch variants directed against Y oligosaccharide are found to mediate ADCC (antibody-dependent cell mediated cytotoxicity) with human and murine effector cells, its IgG3 and IgG2a isotypes are highly active in CDC, (complement-dependent cytotoxicity) and both efficiently inhibit tumor growth in xenografted nude mice (11-13).

BODY

1. Antibodies to LeY antigen.

We have recently examined the recognition properties of BR55-2 conferring Y specificity (Appendix). Epitope mapping experiments indicate that BR55-2 mimics some of the salient features of the lectin IV protein of Griffonia simplicifolia binding to LeY. In contrast to lectin binding to the difucosylated lactoseries structures, the immunodominant portion of the antigen detected by BR55-2 is specific for the type 2 LeY determinant but not the type 1 determinant, Le^b. To elucidate the molecular

recognition properties of BR55-2, molecular modeling was used to build a three-dimensional structure of the antibody variable region. The crystal structure of the lectin IV protein in complex with a Le^b tetrasaccharide unit was used as a guide to probe the molecular basis for BR55-2 antigen recognition and specificity. A conservative approach in placing the Y tetrasaccharide structure was taken in which functional group contacts are conserved on the Le^b/Y structure as observed in GS4. The restraints imposed based on this criteria provided a model consistent with that observed for GS4 binding to the lactoseries structure. Models of BR55-2 indicate that they are representative of the groove type architecture suggested some time ago for anti-carbohydrate antibodies. Current procedures for predicting ligand-antibody interactions is limited, mainly due to the conformational flexibility of ligands and the role of solvent in mediating ligand recognition and binding. However, modeling does provide an avenue for hypothesis testing when relevant alternative structural or binding information is available. In the present case, structures from lactoseries reactive antibodies and the crystal structure of GS4 have defined potential sites on Lewis structures that dictate their specificity.

Comparison of calculated and NMR derived conformers of precursors of type 1 (Galβ1→3GlcNAc) and type 2 (Galβ1→4GlcNAc) chains show that they display very similar topographical features. Spohr et al. (14) have shown that the binding of these structures by GS4 should occur at the termini of the molecule involving the common surface to both structures based on a wide number of chemically modified structures related to Le^b and Y human blood group determinants. The modeled structures for Y binding to the antibodies appear to reflect salient features for binding of Le^b to GS4 and is consistent with antibody reactivity profiles to the lactoseries analogues. Important functional group contacts observed in GS4 are largely conserved in the antibodies, however, some of the interactions differ among the residues and may define their overall specificity. All the sugar moieties (aGlcNac, bGal, cFuc, and dFuc) are stabilized by hydrogen bond and hydrophobic interactions. The residues that stabilize cFuc, dFuc, and bGal are conserved in the BR55-2 and B3 prototypes. Residues Ser31La Asn31Lb, stabilize both bGal and dFuc moieties through bifurcated hydrogen bonds. The cFuc moiety is stabilized by His 93L and the backbone NH group of Val 94L. For the fucose moieties, mimicry of GS4 binding is clearly evident in the role played by Asn L31b and Trp H98 of B3, in that these residues are both preserved in GS4 in contacting the respective functional groups.

Based on the oligosaccharide specificity, lectins and antibodies that distinguish between the difucosylated type 1 and 2 lactoseries structures, it appears that a critical recognition feature for type 1 and 2 chain specificity is an involvement of the 6-CH_2OH and/or acetamido group of GlcNAc residue in the antibody combining site. The involvement of the β-D-N-acetylglucosamine residue in the binding epitope for Y specific MAbs was previously postulated (1). In BR55-2 model, the 6-CH_2OH of GlcNAc interacts with a Ser residue (position 56H) in BR55-2. In H18A, this hydrogen bond is replaced by hydrophobic interactions with Ile 56. We also find that the N-acetamido group forms both polar and nonpolar interactions. The acetamido group in GlcNAca is neutralized by His 58 in BR55 and by Ala in B3 and Tyr in H18A. These differences probably translate into different affinities for Y for these antibodies. In the crystal structure of GS4, GlcNAc is completely exposed and not involved in any interactions. Thus, the GlcNAc moiety is postulated to play a key role in the recognition of Y by the antibodies, while retaining interactions which are shared with GS4. Subsequently, the interaction of the βDGlcNAc residue with Y specific antibodies is a driving mediator in their specificity.

Structural studies on Lewis antigens have generally substantiated that conformations are determined mainly by steric repulsion brought about by changes in the glycosidic dihedral angles. Molecular dynamics calculations on Lewis antigen structure prototypes indicate the lack of spontaneous conformational transitions to other minima during the simulations, suggesting that these oligosaccharides maintain well-defined conformations with relatively long lifetimes (15). These results further indicate that hard sphere or rigid-geometry calculations, albeit in the absence of solvent, provide a good picture of the steric repulsions that modulate the conformational properties of the Lewis antigens. Our minimizations, starting from binding mode geometrys, were found to converge to such conformations. However, it appears that the Y structure might also fluctuate about a binding mode conformation that deviates from the well-defined conformations. The principle deviation lies in the dFuc(1→2)bGal glycosidic torsion angles. In our minimizations for the LeY structure we find that the energy difference

between the converged local minima derived from the antibody binding modes is only 0.1 Kcal/mole. In contrast, we found that analogous Le^b structures are 8 Kcal/mole above these low energy conformers which are typically not observed from previously reported MD calculations (15) and our own calculations.

The interplay between carbohydrates and proteins is of fundamental importance in a number of different biological processes. In particular, cell adhesion and cell recognition events are often mediated by protein-carbohydrate interactions forming a basis in the etiology of tumors. The inhibition of these interactions is a possible point of therapeutic intervention. An understanding of the three-dimensional basis for the molecular recognition of Y by these antibodies can be applied for future diagnosis in tumor progression and micrometastasis as well as active immunotherapy. For example, the model of BR55-2 emphasizes the role played by the fucose methyl groups facing the floor of the antibody combining site. These methyl groups might be mimicked by methyl groups containing amino acid residues in a designed peptide or anti-idiotypes that compete with Y for anti-Y antibodies. A peptide that mimics the Y antigen has been described (16) that contains such methyl groups.

The use of peptides or anti-idiotypes that mimic carbohydrate epitopes might also serve as a unique vaccine strategy for induction of anti-tumor immune responses. Peptides that mimic carbohydrates are shown to be capable of inducing anti-carbohydrate immune responses (17). A peptide or engineered antibody mimic that expresses three-dimensional shapes resembling structural epitopes of the LeY carbohydrate antigen might be recognized as a foreign molecule, breaking any tolerance to human self-antigens (18-21). Further structural studies of Y antigen forms binding to anti-Y antibodies will provide information relevant to vaccine design strategies and improved immunotherapeutics.

2. Adhesion of SkBr5 cells to cytokine activated human endothelial cells.

Type 2 lactoseries structures Y and X are present on the same glycoproteins isolated from MCF-7 mammary adenocarcinoma cells as determined using Y-specific BR55-2, BR15-6A and WGHS-29-1, $D_1$56-45 MAbs, respectively. It was determined that Lewis X (LeX) carbohydrate determinant serves as a ligand for the selectin family of adhesion molecules on cytokine activated endothelial cells and thus is postulated in metastasis of tumor cells. Recently, LeY was demonstrated to mediate tumor cell motility and adhesion to endothelial cells. We have determined that the LeX determinant is associated with all glycoprotein species identified as carrying LeY determinant. This suggests that the isolated glycoproteins of mammary adenocarcinoma, in addition to mediating the cytotoxic function of MAb, are the major carriers of ligands such as X and Y carbohydrate determinants for adhesion molecules, and thus they facilitate the metastatic process. It was previously determined that metastatic potential of the tumor cells correlates with the amount of ligands for E-selectin such as SA-LeX and X determinants associated with lamp-1 glycoprotein, which is present in LeY active glycoproteins isolated from breast carcinoma cells. Thus, it can be postulated that other, yet uncharacterized components of LeY and X reactive glycoproteins in analogy with lamp glycoproteins are also utilized in carbohydrate-selectin interaction of tumor cells to endothelial cells.

We examined the ability of MAbs specific for the Y carbohydrate ligand to inhibit adhesion of colon and breast adenocarcinoma cell lines to cytokine-activated endothelial cells.

Colon adenocarcinoma. We confirmed previous findings that colon carcinoma-derived cell lines attach to endothelial cells and that ELAM-1 specific MAb has the ability to inhibit this interaction. The effect of anti-ICAM and anti-VCA MAbs was much less prominent and the antibody treatment did not have a significant effect on the adhesion. MAbs specific for E-selectin carbohydrate ligands as LeX (CD15), sialylated-LeX and sialylated-Le^a which were previously identified as colon adenocarcinoma-associated antigens were not as effective as E-selectin specific MAb. This is in agreement with other reports demonstrating that among various MAbs with similar specificity, with respect to the recognition of carbohydrate structure, only some did not block the adhesion. It is highly likely that these MAbs bind to different epitopes on CD15 and SA-Le^a. It implies that specific epitopes of the same carbohydrate structure and/or MAb avidity are crucial factors in adhesion. Adhesion of colon adenocarcinoma cells

was not inhibited by the pretreatment of tumor cells with Y carbohydrate specific MAbs BR55-2 and BR15-6A, although Y determinant is strongly expressed in colon adenocarcinoma cell lines and tumors.

Carbohydrate mediated adhesion of adenocarcinoma cells. Adhesive interactions of tumor cells with the luminal side of the lymphatic and vascular endothelium are thought to be an important step in the metastatic process. Tumor cell adhesion to endothelial cell monolayers are investigated in vitro as a model for metastatic invasion. E-selectin, an inducible endothelial cell-surface glycoprotein, is implicated to mediate adhesion of colon, lung, and ovary carcinoma cells to cytokine activated endothelial cells. The molecular mechanisms of the entrapment of breast adenocarcinoma cells via carbohydrate determinants are basically unknown. Our preliminary data suggest that various mammary adenocarcinoma cells attach to cytokine-activated HUVEC via two ways, E-selectin dependent and independent modes. Estrogen-sensitive MCF-7 cells adhere to TNF stimulated EC and this process is mediated by E-selectin as determined by the inhibitory effect of anti-E-selectin MAb. The treatment of carcinoma cells with MAbs BR55-2 and BR15-6A specific for α-2-fucosylated structure LeY significantly decreased the adhesion in a dose dependent fashion (data not shown), indicating that LeY is involved in adhesion. Since this structure has not been identified so far as a selectin ligand, these results suggest that this interaction is independent of E-selectin or that E-selectin might recognize an epitope on the LeY structure. Both LeY and LeX represent biosynthetically and conformationally related structures.

In support of the notion that an E-selectin independent pathway exists, we have observed other human breast adenocarcinoma cell lines, SkBr5 and SkBr3, but not colon adenocarcinoma cells LS180, SW1116 and SW948, to have the capacity to adhere to the spontaneously transformed human endothelioma cell line ECV-304 cells (Fig. 1) which does not express E-selectin even in the presence of cytokines as assayed by RIA using anti-selectin antibodies (Fig. 2). These results indicate that the adenocarcinoma lines interact with ECV-304 cells independently of E-selectin. Despite the lack of E-selectin expression, 50% (IC 50) attachment of SkBr5 and SkBr3 cells was observed in the presence of MAb NS19-9 which is specific for the selectin ligand SA-Le^a. Antibodies BR55-2 and BR15-6A directed to LeY showed a similar level of inhibition in this system as compared to the negative control antibody (H2B45) (Fig. 1).

Figure 1

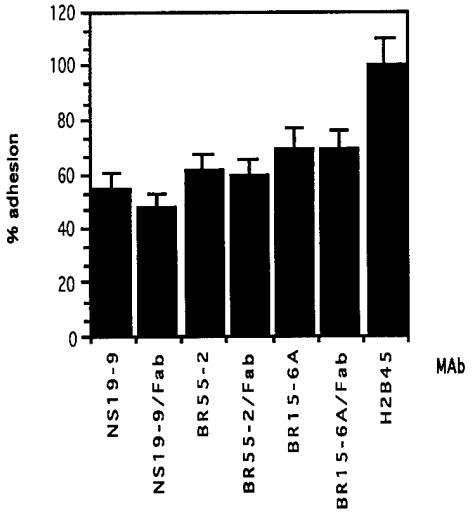


Figure 2

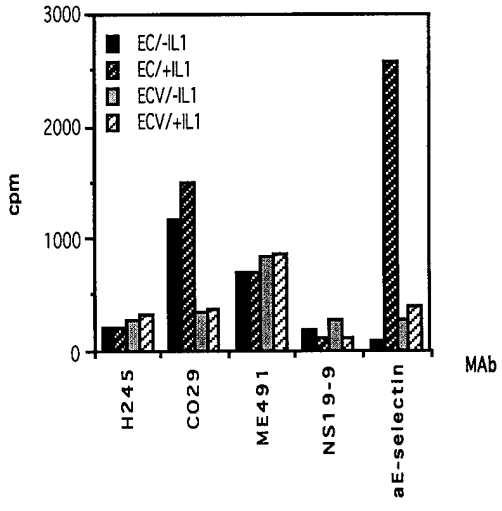


Fig. 1. Adhesion of human mammary adenocarcinoma cells SkBr5 to human endothelioma cell line ECV-304. MAbs and MAb F(ab)$_2$ fragments (marked F in the graph) at 40 μg/ml were kept during the 15 min adhesion of SkBr5 and HUVEC. Control anti-influenza MAb H24B5 was used at the same concentration. The results represent % of adherent cells in the presence of specific MAb as compared to control anti-influenza heamaglutinin MAb H24B5. Results are reported as means ± one standard error of five independent experiments.

Fig. 2. Representative data of various antigen expression as determined by MAbs binding in RIA with monolayer ECV-304 cells. SA-Le^a (MAb NS19-9), CD63 (MAb CO29 and ME491), E-selectin specific MAb and negative control MAb H24B5.

Furthermore, small cell lung cancer (SCLV) cell lines that express large quantities of lactoseries structures such as LeX, LeY and H-2, bind to endothelioma cells as opposed to SCLV cells that lack expression of these structures. These findings strongly suggest the existence of a novel carbohydrate mediated, yet independent of the classic selectin pathway, involved in adhesion of human carcinoma cells to endothelium involving fucosylated lactoseries structures. A recent report identified highly invasive mammary and colon adenocarcinoma cells which do not interact under dynamic conditions, but adhere to resting and stimulated EC in static adhesion phase that was independent of E-selectin (22). These observations highlight important differences among cancer cells in their mode of interaction with endothelium based on carbohydrate-protein interactions and strongly suggest a non-selectin mediated pathway for some breast adenocarcinoma adherence.

The induction of anti-carbohydrate immune responses by peptides. We have shown that a synthetic peptide can mimic the capsular polysaccharide of *N. meningitis* serogroup C (MCP) in that it induces an anti-MCP immune response (17). This peptide was derived from an anti-idiotypic antibody which was capable of inducing an anti-MCP idiotype. Subsequently, we showed that a large immunogen can be reduced to a smaller fragment that retains its biological activity. Furthermore, this peptide complexed to proteosomes was found to protect Balb/c mice from lethal infection of *N. meningitis*. This peptide bears some homology with other peptides that mimic carbohydrates identified from phage display peptide libraries (Table 1). All proteins which interact with α-amylase have WRY residues implicated in this interaction. There are two aromatic residues separated by a charged residue. Recently, Hoess et al. (16) have identified an 8 amino acid peptide expressed on a phage capable of inhibiting LeY carbohydrate-antibody binding consisting of the sequence tract PWLY. A peptide which binds ConA has been shown to have a similar configuration, namely YPY. Both peptides demonstrate the preference of aromatic groups separated by an intervening residue. All these sequences resemble the peptide we have identified as a mimic of the group C meningococcal polysaccharide. Peptides which mimic carbohydrates may follow identical "rules".

Table 1

Peptide	Carbohydrate	Structure
YYPY (P1)	Mannose	methyl-α-D-mannopyranoside
WRY	Glucose	α(1-4)glucose
PWLY	Lewis Y	Fucα1→2Galβ1→4(Fucα1→3)GlcNAc
YYRYD (P2)	Group C Polysaccharide	α(1-9)sialic acid
YYRGD (P3)		

We have further evaluated the ability of sera to P1, P2, and P3 containing peptides to bind to tumor cells as evaluated by FACS assay. We found that murine sera elicited against a couple of these peptides bind to human breast carcinoma and murine melanoma cells expressing a high level of lactoseries structures. The anti-peptide sera to one designed peptide containing the P3 motif was found to bind extremely well to the human breast cancer cell line SkBr3, with the other two peptide sera displaying preferential binding to both tumor lines. Some cross-reactivity of the anti-P3 sera to NIH3T3 cells was observed, but its specificity for SkBr3 was 4 fold higher. In control assays the normal breast cell line HS578 displayed reduced fluorescence.

Table 2 Binding of Various Anti-Carbohydrate Sera to Different Cells As Measured by FACS (Final Sera Concentration: 1:50)

Peptide Cell Line	Anti P_1 (YYPY)	Anti P_2 (YYRYD) Mean Fluorescence	Anti P_3 (YYRGD)
SKBR3	81.6	83.6	166.7
HS578 Bst (normal breast)	21.0	20.9	21.7
B16B78H1	38.0	32.0	28.0
NIH3T3 Murine Fibroblasts	20.9	21.8	41.7

Background fluorescence associated with non-specific mouse sera is 24.2, 25.2, and 23.7 for SKBR3, B16B78H1, and NIH 3T3 cells, respectively.

These data indicate that the general conformational properties are retained by peptides which mimic carbohydrates and can induce polyclonal responses that target cells expressing high levels of carbohydrates. These data also suggest that perhaps mimicking peptides adopt conformations which allow different carbohydrate structures to be mimicked in parallel.

We have further examined the ability of these peptides to induce anti-carbohydrate responses such that they might neutralize HIV. We have immunized Balb/c and C57/BL6 mice with peptide derivatives of P1, P2, and P3 and have found that derived anti-P1 and P2 sera and monoclonal antibodies can block HIV infection. Sera was prepared from mice immunized 3 times with 15, 25 or 50 μg peptide-proteosome complex/mouse. These antisera and monoclonals were reactive with meningococcal group C-Polysaccharide (MCP) (17). For neutralization, the amount of infectious virus (50% tissue culture infective dose, i.e., $TCID_{50}$) present in supernatants from infected cells was measured following infection. To determine if the immune sera or monoclonal antibodies can neutralize the virus, 50 μl of supernatant, containing 100 TCID/50 of HIV-I were preincubated with 50 μl of serial dilutions of antisera, monoclonal antibodies, or the controls (normal human sera (NHS) or mixture of 4 sera of HIV-I positive patients) for one hour at 37°C. After incubation, 50 μl of target cells (HTLV-I/MT-2) were added and cultures were incubated for an hour at 37°C. Neutralization was detected as inhibition of syncytia and/or by detection of cell proliferation using the MTT assay. The sera, which were collected from mice one week after the last injection, were used for neutralization of HIV-1 infection at final dilutions of 1:16; 1:32; and 1:64. The sera from Balb/c (H2d) and C57Bl/6 (H2b) mice immunized with P1 or P2 (25 and 50 μg, but not 15 μg/mouse), at dilutions up to 1:64, neutralized both HIV-1/MN and 3B strains (data not shown). Fig. 3a shows representative data of neutralization of HIV-1/MN for anti-P1 and P2 sera from Balb/c mice immunized with 25 μg peptide/mouse: NHS, as well as the anti-P3 sera, were ineffective at blocking syncytia formation, whereas human a-HIV-1 sera from infected patients was very effective. The MTT assays corroborated these data (data not shown). These results suggest that the production of HIV-neutralizing antibodies in this system is not MHC class-restricted, but is dose-dependent and is as effective as sera from HIV-1 infected patients.

Monoclonal antibodies against the above-mentioned peptides were prepared. Supernatants from these monoclonals were added to neutralization assays at final dilutions of 1:3, 1:15, and 1:75. Fig. 3b shows that monoclonal antibodies against P1 and P2, but not against P3, were effective at inhibiting cell-free viral infection (HIV-1/3b strain). These distinctions are consistent with those shown with polyclonal sera. We have examined whether the polyclonal anti-peptide sera binds to glycosylated gp140 env protein of HIV-1 MN, lacking the transmembrane domain of gp41. As presented in Fig. 4, ELISA indicates the three anti-peptide sera bind to this fragment in spite of the fact that only two display HIV-1 neutralizing activity. Control groups include a negative Balb/c derived polyclonal sera directed to an irrelevant antigen, and a positive control Balb/c monoclonal directed to HIV-1 unglycosylated gp120. We are presently testing unglycosylated gp120/gp140 binding to these sera.

Figure 3. Neutralization of HIV-1/MN by anti-peptide polyclonal sera (1a) and HIV-1/IIIb by anti-peptide monoclonal sera (1b).

Figure 3a

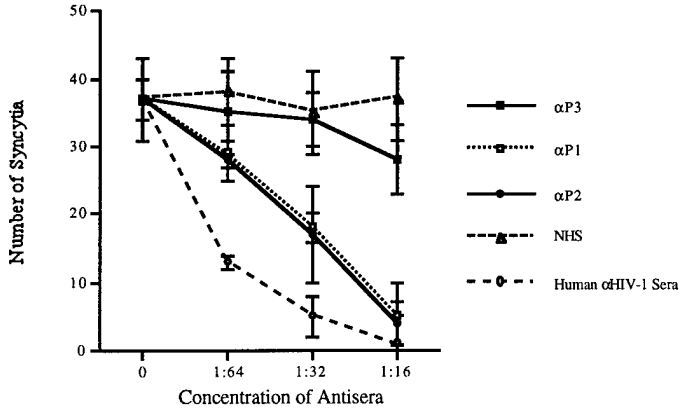


Figure 3b

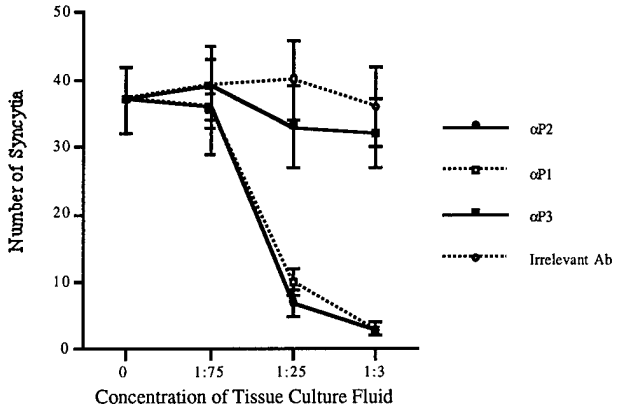
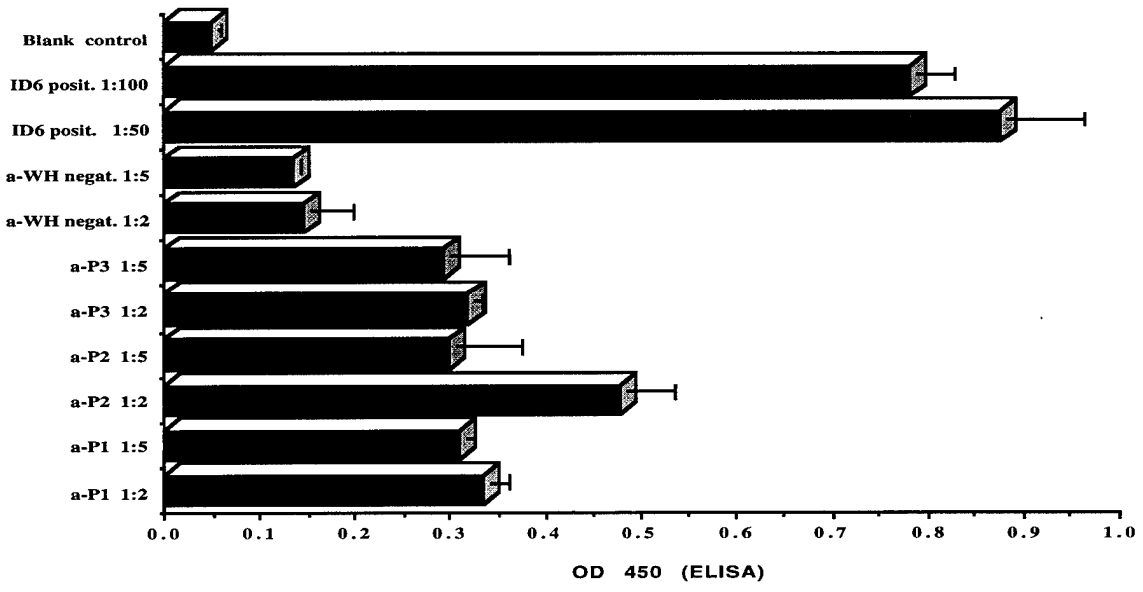


Figure 4. Binding of anti-peptide sera to glycosylated GP140 HIV-1/MN.



Peptides as mimics of carbohydrates. We have further characterized peptides derived from a 15-mer phage display peptide library for binding to anti-Y antibodies and we will extend this approach to antibodies binding to SA-LeX, LeX SA-Le^a as discussed in this application. The choice of using the 15-mer library was predicated on the notion that this length is similar to complementarity determining regions (CDR) in antibodies which confer mimicry capacity to many so-called anti-idiotypic antibodies. Most recently, we have shown that a CDR1 derived peptide from the light chain of a recombinant antibody mimics a bioactive region on GM-CSF (23). This peptide has the sequence CRASKSVSSSGYSYMHWYQQ and mimics what appears to be a 3-dimensional epitope on GM-CSF that involves two helices. This peptide was shown to display GM-CSF activities (23). The putative active residues include the sequence tract SKSVSSS. The recombinant antibody was identified by screening against an anti-GM-CSF neutralizing antibody 126.213 (23).

To validate the notion that a 15-mer library should contain peptides representative of CDR domains, we have screened the 15-mer peptide library using 126.213. Analysis of families of sequences include the following: Family 1, GAPIFPVVSSSGSSSSPGA; Family 2, GARNVGFGFVTSASGRDGA; and Family 3, GAPPFPFFLSLAHAGPAGA. We observe that the putative sequence VSSSGYSY is mimicked by Family 1, displaying the sequence tract VSSSGSSS in which the OH- functional groups on the YSY tract in the CDR1 peptide of the recombinant antibody are preserved by the SSS tract in the Family 1 sequence. A peptide spanning the SSS sequence tract was shown to inhibit 126.213 binding to the recombinant antibody (data not shown). These results verify that the 15-mer peptide library is representative of CDR lengths.

We have recently sequenced phages that bind to two anti-Y antibodies, BR55-2 and BR15-6A. Families for BR55-2 include the following sequences: Family 1, GAHGRFILPWWYAFSPSGA; Family 2, GATWPVVHGACRAHGHCGA; Family 3, GATSVNRGFLLQRVSHPGA; Family 4, GAGRVASMFGGYFFFSRGA; Family 5, GARYLFYSVHPWRVSYSGA; Family 6, GAGLDLLGDVRIPVVASGA; Family 7, GAWPYLRFSPWVVSPLGGA; Family 8, GAGAFSSPRSLTVPLRRGA; Family 9, GASLVSSLDIRVFHRLPGA; Family 10, GAARVSFWRYSSFAPTYGA; Family 11, CAFARYLFTHWWRLPVDGA; Family 12, GAVGITGFVDPLPLRLLGA; Family 13, GAARFRHSTKSAQFVPLGA; Family 14, GAIMILLIFSLLWFGGAGA. Families for BR15-6A partially overlap with those identified with BR55-2 and include: Family 1, GAHGRFILPWWYAFSPSGA; Family 2, GATVGASFWWLSGGKVPGA; Family 3, GAGLDLLGDVRIPVVRRGA; Family 4, GALAFVWTVAVPPFPPGGA. These sequences can be compared with a putative peptide identified by phage display panning with another anti-Y antibody, B3 (16). In that study, a peptide with the sequence APWLYAGP was observed to bind to B3 and inhibit B3 binding to the Y carbohydrate structure. The tumor cell binding specificities of B3, BR55-2 and BR15-6A are different. We have tested the APWLYAP peptide for binding to BR55-2 and BR15-6A which proved negative. Nevertheless, the central Planar residue-X-Planar residue tract is represented in Families 1, 4, 5, 7, and 10 for BR55-2 and observed in Families 1, 2, and 4 for 15-6A. In Family 7, identified by BR55-2, an inverted tract AWPYL is observed in comparison to the sequence APWLY found by B3. A sequence tract of WRY associated with α(1-4) glucose structure is observed in Family 10. It is of interest to note that a peptide (YYWIGIRK) derived from the lectin domain of E-P- and L-selectins blocks selectin-mediated cell adhesion. Subsequently, it may turn out that Planar and hydroxyl groups containing peptides displayed in a variety of ways can lead to inhibition of cell adhesion either by mimicking carbohydrates or by interacting with them. Peptides are presently being made covering the various families based upon structural models of the 15-mer loop in comparison with studies on the CDR1 conformations of the recombinant antibody discussed above. These sequences will also be compared with an anti-idiotype sequence (after completion), E4, which is a mimic for LeY and prepared against BR55-2.

As a consequence of these studies we have developed a chemiluminescence approach to sequencing which utilizes a biotin labeled at the 5' end of the primer (manuscript in preparation). The primer is then purified, and using delta Taq PCR cycle sequencing method, a 6% denaturing sequencing gel is run. We then transfer all DNA bands to nylon membrane and block the membrane. Developing solution is then added. We leave the membrane at room temperature for 2 hours in the dark, after which

it is ready for film exposure. This membrane can emit a stable signal for several days. Film is exposed to the membrane for 15-30 minutes. This approach is both rapid and uses non-radioactive material, providing a very sensitive method for sequencing. We are now examining this approach for sequencing antibody libraries reactive with LeY antigen.

CONCLUSIONS

The interplay between carbohydrates and proteins is of fundamental importance in a number of different biological processes. In particular, cell adhesion and cell recognition events are often mediated by protein-carbohydrate interactions forming a basis in the etiology of tumors. Aberrant glycosylation may be crucial in tumor progression, since cells acquire competence for metastasis and a faster clonal growth via newly synthesized carbohydrate structures (24). The carbohydrate expression patterns differ according to clinical features and are also changeable in clinical courses of respective tumor types (25,26). The differential exposure at the cell surface of specific carbohydrates may have implications for cell-protein or cell-cell interactions (27-29) and for antibody-directed tumor detection and therapy. To date, the functional role of the Lewis antigens have not been fully explored. Presumably, these carbohydrates represent functional structures or determinants of molecules controlling cell motility, adhesion, and proliferation, functions related to the metastatic potential of human cancers. Our preliminary adhesion studies strongly suggest the existence of a novel carbohydrate pathway (selectin independent) involved in adhesion of human carcinoma cells to endothelium involving fucosylated lactoseries structures. These data also suggest that perhaps some overlap in conformations among carbohydrate structures can exist.

Antibodies are known to function as surrogates or mimics of ligand binding sites on receptors, binding to both receptor agonists and antagonists. Anti-Idiotypes to BR55-2 can induce anti-Y responses. We chose BR55-2 and BR15-6A antibodies for phage screening to further identify potential relationships among peptides that mimic carbohydrates, and since it appears that the carbohydrate ligands of interest in this proposal are also able to modulate cell adhesion in a non-selectin mediated pathway. Thus, antibodies provide a useful means to identify reagents that inhibit both selectin and non-selectin adhesion molecules since selection is based on the conformational properties of the ligand and not the adhesion molecules themselves. We have identified several peptide families binding to BR55-2 and BR15-6A whose sequences compare well with peptides to mimic carbohydrate structures. We have shown that we can induce anti-carbohydrate immune responses that target tumors. We are also presently sequencing anti-Ids to BR55-2 and will compare these sequences with the identified peptides from the phage screening. In the coming year we will synthesize representative peptides and test their ability to induce anti-Y responses and to inhibit cell adhesion of adenocarcinoma breast cells. In addition, we are using phage display technology to identify human antibodies that react with Y and might prove useful as reagents to target Y in passive therapy approaches. We will compare such sequences with those found by humanizing BR55-2.

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Molecular recognition of the Lewis Y antigen by monoclonal antibodies

Running Titile: Monoclonal antibody recognition of the Y antigen

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Abstract

The murine monoclonal antibody BR55-2 is directed against the tumor associated antigen Lewis Y oligosaccharide. Epitope mapping experiments indicate that BR55-2 mimics some of the salient features of the lectin IV protein of Griffonia simplicifolia binding to Lewis Y. In contrast to lectin binding to the difucosylated lactoseries structures, the immunodominant portion of the antigen detected by BR55-2 is specific for the type 2 Lewis Y determinant but not the type 1 determinant, Lewis b. To elucidate the molecular recognition properties of BR55-2, molecular modeling was used to build a three-dimensional structure of the antibody variable region. The crystal structure of the lectin IV protein in complex with a Lewis b tetrasaccharide unit was used as a guide to probe the molecular basis for BR55-2 antigen recognition and specificity. Docking of a tetrasaccharide Lewis Y structure to BR55-2, indicated similarities in binding topographies between BR55-2 and the lectin IV protein. Conformational energy calculations suggest that selectivity for Lewis Y over Lewis b structures by BR55-2 reflect energetic differences in binding mode conformations of the difucosylated structures. We observe that a major source of specificity for the Lewis Y structure by anti-Y antibodies emanates from interaction with the β -D-N-acetyl-glucosamine residue.

Introduction

Cell-surface carbohydrates play a role in tumor growth, progression and metastases (Hakomori 1989; Hakomori 1991). Among the carbohydrate types, the blood group Lewis antigens are highly associated with a number of cancers including human breast, colon, lung and ovarian carcinomas (Fukushi, Orikasa et al. 1986; Itzkowitz, Yuan et al. 1986; Kim, Yuan et al. 1986; Blaszczyk-Thurin, Thurin et al. 1987; Hoff, Matsushita et al. 1989; Hoff, Irimura et al. 1990; Cooper, Malecha et al. 1991; Tsukazaki, Sakayori et al. 1991; Itzkowitz 1992; Miyake, Taki et al. 1992; Murata, Egami et al. 1992; Ogawa, Inoue et al. 1992; Ichihara, Sakamoto et al. 1993; Iliopoulos, Atkinson et al. 1993; Kobayashi, Sakamoto et al. 1993). The Lewis Y (Y) difucosylated type 2 lactoseries structure, expressed on both glycoproteins and glycolipids, is one tumor associated carbohydrate structure being explored as a target for monoclonal antibody (MAb) based imaging and therapy (Trail, Willner et al. 1993; Choe, Webber et al. 1994). It is postulated that the Y determinant is of key importance for tumor cell growth or maintenance since it mediates internalization and killing of Y specific MAb (Hellstrom, Garrigues et al. 1990; Pai, Batra et al. 1991; Steplewski, Lubeck et al. 1991; Pai, Batra et al. 1992; Schreiber, Hellstrom et al. 1992; Garrigues, Garrigues et al. 1993).

While a large number of anti-Y antibodies have been generated, their detailed specificity has been studied in only a few cases (Blaszczyk-Thurin, Thurin et al. 1987; Hellstrom, Garrigues et al. 1990; Pastan, Lovelace et al. 1991; Kitamura, Stockert et al. 1994). We previously described a monoclonal Ab, BR55-2, generated against a human gastric adenocarcinoma cell line, that specifically recognizes the Y determinant (Blaszczyk-Thurin, Thurin et al. 1987; Steplewski, Blaszczyk et al. 1990;

Scholz, Lubeck et al. 1991; Steplewski, Lubeck et al. 1991). Monoclonal BR55-2 (IgG3) and its isotype switch variants are found to mediate both antibody-dependent cell mediated and complement-dependent cytotoxicity and efficiently inhibit tumor growth in xenografted nude mice (Steplewski, Blaszczyk et al. 1990; Steplewski, Lubeck et al. 1991). Humanized or chimeric forms of anti-Y antibodies that share some of the properties of BR55-2 are being considered for passive therapy (Kaneko, Iba et al. 1993; Kitamura, Stockert et al. 1994). Subsequently, an increased understanding of the structural basis for antibody recognition of the Y antigen might be exploited to develop improved diagnostic agents and passive and active immunotherapeutics for Y expresing solid tumors.

NMR studies of synthetic Y and Le^b structures show that the major differences in topography between Le^b and Y molecules is provided by the change of glycosidic linkage from $\beta1\rightarrow3$ to $\beta1\rightarrow4$ in type 1 and 2 chains, respectively (Lemieux, Bock et al. 1980; Thogersen, Lemieux et al. 1982; Lemieux and Bock 1983; Hindsgaul, Khare et al. 1985; Rao and Bush 1988; Cagas and Bush 1990; Cagas and Bush 1992; Chai, Hounsell et al. 1992; Strecker, Wieruszeski et al. 1992). This results in conformers in which the N-acetyl and hydroxymethyl groups of the GlcNAc moiety are projected on opposite sides of the type 1 and 2 structures (Figure 1a). Functional groups that are shared or define the common topography between the type 1 and type 2 structures account for their mutual recognition by GS4 (Spohr, Hindsgaul et al. 1985). The immunodominant portion of the antigen detected by BR55-2 is specific for the type 2 Y determinant but not the type 1 isomeric determinant, Lewis b (Le^b). This type 2 specific recognition is in contrast to the lectin IV protein of Griffonia

simplicifolia (GS4), suggesting that BR55-2 mimics only a portion of the salient recognition features of GS4.

The aim of the present study was to characterize the dimensions and possible similarities of BR55-2 with other anti-Y antibodies, and with GS4 in contacting the Y determinant. Current methods are insufficient to allow the detailed prediction of antibody-antigen interactions or the conformational changes induced in antibodies or ligands upon antigen binding (Cheetham, Raleigh et al. 1991; Rini, Schulze et al. 1992). However, potential putative contacts between BR55-2 and Y might still be assessed by considering the crystal structure of the lectin IV protein of Griffonia simplicifolia (GS4) complexed with Le^b (Vandonselaar, Delbaere et al. 1987; Delbaere, Vandonselaar et al. 1993). Studies of antibody-mimics of ligand binding sites suggests that mimicry can be achieved by antibodies contacting similar ligand functional groups as a native receptor, albeit perhaps presented in a different molecular configuration (Williams, Moss et al. 1989; Williams, Kieber-Emmons et al. 1991; Williams, Kieber-Emmons et al. 1991; Lin, Kieber-Emmons et al. 1994). Analysis of BR55-2 for Y binding provides a further case to elucidate the ability of antibodies to behave as surrogate ligand binding sites.

Molecular modeling of BR55-2 was used to identify potential antibody interaction schemes with the putative tetrasaccharide units of the Y determinant and compared with other anti-Y antibodies, B3 (Brinkmann, Pai et al. 1991; Pai, Batra et al. 1991; Pastan, Lovelace et al. 1991; Pai, Batra et al. 1992; Choe, Webber et al. 1994), H18A (Kaneko, Iba et al. 1993) and BR96 (Garrigues, Garrigues et al. 1993; Trail, Willner et al. 1993; Bajorath 1994; Garrigues, Anderson et al. 1994). The BR55-2 model emphasizes key polar and nonpolar interactions between the

antibodies and the oligosaccharides, sharing the salient features of GS4 binding to the lactoseries carbohydrates. A molecular model of the antibody BR96 has been presented (Bajorath 1994) that is consistent with the model presented here, but lacks the detailed recognition features for Y identified herein. In agreement with conclusions drawn from consideration of reactive profiles of lactoseries oligosaccharide probes with BR55-2, we observe that a major source of differential specificity for the type 1 and 2 difucosylated structure emanates from antibody interaction with the β-D-N-acetyl-glucosamine residue as compared to GS4 and that binding modes for Y to BR55-2 are high energy structures for Le^b.

Materials and Methods

Cloning and sequencing of the heavy chain and light chain variable domain cDNA for BR55-2

The variable domain cDNA for the heavy chain and light chain of BR55-2 was cloned by the anchored PCR method (Co, Avdalovic et al. 1992). First, a total RNA preparation was prepared using the hot phenol method. Briefly, $1x10^7$ BR55-2 hybridoma cells were resuspended in 1.2 ml of RNA extraction buffer (50mM sodium acetate, pH 5.2 , 1% SDS), vortexed and incubated at room temperature for 2 min. The cell lysates were then incubated with 0.6 ml of phenol, pH 5.2, at 65^o for 15 min, followed by another 15 min incubation on ice. The extract was spun in a microfuge; the aqueous phase was recovered and ethanol precipitated twice. The RNA pellet was resuspended in water and quantitated at OD_{260}. cDNA was synthesized from the total RNA using reverse transcriptase (5 ug total RNA, 40 ng dT12-18 (Pharmacia), 200 units of M-MLV reverse transcriptase (BRL), 40 units of RNAsin (Pomega), 50 mM Tris-HCl, pH 8.3,

75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$ and 0.5 mM each dNTP in a 20 ul reaction volume). The G-tailing was achieved with terminal deoxynucleotidyl transferase (TdT) (cDNA, 15 units TdT (BRL), 0.1 M potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM DTT and 1 mM dGTP in a 20 ul reaction volume). Under the conditions described, tails generally contained about 20 bases. One half of the G-tailed product was then amplified to generate the V_L gene and the other half amplified to generate the V_H gene, using Taq polymerase. The V_L gene was amplifed with the primer TATATCTAGAATTCCCCCCCCCCCCCCC that anneals to the G tail and a primer TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC that anneals to the constant region of the kappa light chain. The V_H gene was amplified with the same C tail primer and a primer TAGAGCTCAAGCTTCCAGTGGATAGAC(CAT)GATGGGG(GC)TGT(TC)GTTTTGGC that anneals to the C region of gamma chains. The sequences in parentheses indicate base degeneracies, which were introduced so the primer would be able to recognize all gamma chain isotypes. EcoRI and HindIII sites are included in the upstream and downstream primers for convenient subcloning into pUC18 vector. An alternate set of restriction sites (XbaI and SacI) are also included in the primers for the rare event that EcoRI and HindIII sites are present in the variable region genes. The PCR reactions were performed in a programmable heating block using 30 rounds of temperature cycling (92^{o}C for 1 min, 50^{o}C for 2 min and 72^{o}C for 3 min). The reaction included the G-tailed product, 1 ug of each primer and 2.5 units of Taq polymerase (Perkin Elmer Cetus) in a final volume of 100 ul, with the reaction buffer recommended by the manufacturer. The PCR product bands were excised from a low-melting agarose gel, digested

with restriction enzymes and cloned into pUC18 vector for sequence determination.

Modeling of BR55-2

It is now recognized that the shapes of heavy and light chains are closely associated with the lengths of complementarity determining regions (CDRs) and that framework (Fr) residues play an important role in influencing CDR conformations (Chothia, Lesk et al. 1992; Tramontano and Lesk 1992) Employing this concept, and following procedures we and others have used in previous studies on antibody modeling (Fine, Wang et al. 1986; Bruccoleri and Karplus 1987; Shenkin, Yarmush et al. 1987; Bruccoleri, Haber et al. 1988; Novotny, Bruccoleri et al. 1990; Cheetham, Raleigh et al. 1991; Martin, Cheetham et al. 1991; Mas, Smith et al. 1992; Nell, McCammon et al. 1992; Tomiyama, Brojer et al. 1992; Lohman, Kieber-Emmons et al. 1993; Bajorath 1994; Lin, Kieber-Emmons et al. 1994) the conformations of the variable domains of BR55-2 was deduced by comparison with known immunoglobulin crystal structures (Bernstein, Koetzle et al. 1977). Sequence and crystallographic structure comparisons were used to identify templates for the localized structural folds of the hypervariable or CDR1, CDR2 and CDR3 regions of the heavy and light chains. For modeling of the heavy chain CDR3 region in particular, a knowledge based approach was considered (Blundell, Sibanda et al. 1987) to search for suitable structures that fit a geometry to the base residues of the CDR3 domain (Fine, Wang et al. 1986; Shenkin, Yarmush et al. 1987; Martin, Cheetham et al. 1991) as opposed to calculating the loops through conformational search procedures (Bruccoleri, Haber et al. 1988; Novotny, Bruccoleri et al. 1990; Mas, Smith et al. 1992; Nell, McCammon et al. 1992)

To determine this base geometry, the Cα positions of several antibodies of known crystal structure were superimposed to define invariant residue positions. These positions define the amino-terminal beginning and the carboxyl terminal end that are shared among the putative CDR3 domains of varying lengths (Tomiyama, Brojer et al. 1992; Karp, Kieber-Emmons et al. 1993; Lohman, Kieber-Emmons et al. 1993; Lin, Kieber-Emmons et al. 1994). The systematic superpositioning of the CDR3 domain over short sequences define a consensus region where the base geometry is conserved among the antibody templates. The description of invariant positions effectively reduces the length of the loop to search to those that are representative of a sufficient saturation of conformational space in the crystallographic database; usually six to seven residues in length. The crystallographic database was searched to identify loops of the same size as the CDR3 loop being examined using the program InsightII (Biosym Technologies version 3.5). The spatially conserved cartesian positions of the the N and C terminal regions of CDR3 were held fixed in this search procedure. The 10 best matches were examined using the program InsightII and an appropriate choice was made based upon similarities in positions of side chains at the junctures of the CDR3 loop.

The CDRs and the FRs of the templates were mutated to those of the respective antibody heavy and light chains using Insight II. The side chain angles of the substituted residues were set according to angles identified in a database of side chains. Each CDR and framework region was changed individually, followed by 1000 cycles of energy minimization to eliminate close contacts between atoms. As in our previous studies, the program Discover (version 2.95 Biosym Technologies) was used for conformational calculations with the supplied consistent valence force field (CVFF)

parameters. After model building, the respective structures were energy optimized to convergence. Molecular dynamics (MD) at 300K° and 600K° was used to further alleviate any close contacts within the antibodies.

Initially a molecular dynamics simulation over 30 picoseconds using the program Discover was performed. The structure was then energy minimized using conjugate gradients to convergence. Following this initial equilibration, the calculation was resumed for another 20 picoseconds at 600K° at constant pressure and then cooled to 300K° over 30 picoseconds. During the second dynamics procedure atoms lying further than 15 Å from all atoms of the CDR loops were held fixed. Non-hydrogen atoms of residues lying in the region 9 to 15 Å from all CDR loop atoms were harmonically restrained to their initial positions with a force constant of 30 kcal x mol^{-1} x rad^{-2}. These distance approximations result in fixing or restraining atoms of residues within the framework region of the antibodies. The backbone conformation torsion angles, phi and psi, of non-CDR loop residues were restrained to their initial values with a force constant of 1600 kcal x mol^{-1} x rad^{-2}. In addition, a torsional restraint of 10 kcal x mol^{-1} x rad^{-2} was employed around the omega bond. A time step of 1 fs was used. The resulting structure for BR55-2 was energy minimized using conjugate gradients to convergence.

Docking of Lewis Y to BR55-2

Individual subunits derived from crystal structures of carbohydrate fragments, was used to model the putative Y tetrasaccharide core Fucα1→2Galβ1→4(Fucα1→3)GlcNAc structure. The modeled structure was first energy minimized and compared with published computational (Lemieux and Bock 1983; Mukhopadhyay and Bush 1991) and NMR (Cagas and Bush 1990; Cagas and Bush 1992) results on Y and Le^b determinants. Residues

associated with the CDR loops were identified for possible interaction with the Y determinant based upon Lewis antigen recognition by GS4 (Delbaere, Vandonselaar et al. 1993). The approach taken in the placement of the Lewis Y core in the antibody combining site was that described previously by us (Lin, Kieber-Emmons et al. 1994). The binding surface on the modeled antibodies was first defined as sites accessible to probe spheres of varying radius (1.4 to 1.7 Å) to identify the possible positions which can be occupied by the atoms of the Y structure. The probe spheres were rolled on the binding surface of the models, with the continuum of loci reduced to a set of discrete points by clustering neighboring spheres much like a set of site points (Lin, Kieber-Emmons et al. 1994). These site points are localized at atom positions accessible to the probe spheres on each residue on the surface of the antibody. The site points were then used as a guide in the placement of the Y structure. Hydrogen bonding restraints were applied to enhance potential contacts between the respective antibody combining site residues with sugar groups identified in the GS4/Leb structure (Delbaere, Vandonselaar et al. 1993), allowing the glycosidic angles of the Y structure to change and the respective antibody to adjust to these restraints.

After minimization, a restrained molecular dynamics calculation over 100 picoseconds using the program Discover was performed, preserving the hydrogen bonding constraints. The dynamics run was not intended to be a detailed study, but to further alleviate any close contacts within the antibody and between tetrasaccharide and the antibody. The calculation was initialized and equilibrated for 50 picoseconds at 300K° at constant pressure and resumed for another 50 picoseconds. The resulting structure was energy minimized using conjugate gradients to convergence. In the

minimization and dynamics run no constraints other than the retention of Y-antibody hydrogen bonds were placed on the antibody or binding site. Charges and non-bonded parameters for the Y structure were assigned from atom types from the CVFF parameter list supplied with Discover/InsightII.

Systematic Conformational Search of Lewis antigens

Systematic conformational searches over torsional space on both Y and Le^b were performed to relate their binding mode conformations. Utilizing this grid search approach a dihedral angle is stepped through a range of values, and each resulting conformation is accepted if its interatomic distances do not indicate steric clashes between van der Waals radii of the constituent atoms. Conformational search calculations were performed using the program Search and Compare (Biosym Technologies, Version 2.3.5). The respective units in Y and Le^b were searched over conformational space at 1 or 5° intervals either holding phi/psi (φ,ψ) angles for non-moving glycosidic linkages to their binding mode values or allowing all angles to move concomitantly. Two definitions of the glycosidic (φ,ψ) angles were used to correspond to previously published work (Lemieux and Bock 1983; Mukhopadhyay and Bush 1991). The first definition (φ^1,ψ^1) defines the phi (φ^1) angle as H_1-C_1-O-C_a, and the psi (ψ^1) angle as C_1-O-C_a-H_a. The second definition (φ^2,ψ^2) corresponds to the IUPAC convention in which phi is defined as O ring -C_1-O_1-C'_x, and psi is defined as C_1-O_1-C'_x-C'_{x-1}.

Molecular dynamics calculations were also performed for both Le^b and Y tetrasaccharide structures. The structures were first equilibrated at 300K° for 50ps, followed by100 ps of molecuar dynamics at 300K°. A total of 100,000 conformations were sampled with instantaneous

dynamics structures minimized at 1 ps intervals, reducing the number of structures to be examined to 100. The 100 structures were minimized to convergence using conjugate gradients. The differences in populations of the two molecules were analyzed using the Analysis option in InsightII. In vacuo calculations were performed with a dielectric of 1 or 80 to monitor electrostatic effects on final conformations.

Results

Mapping of recognition sites of Lewis binding proteins

Natural oligosaccharides and synthetic probes of Lewis antigens have been used in a variety of studies to map potential recognition sites on lectins and antibodies (Young, Johnson et al. 1983; Lemieux, Wong et al. 1984; Hindsgaul, Khare et al. 1985; Lemieux, Venot et al. 1985; Spohr, Morishima et al. 1985; Blaszczyk-Thurin, Thurin et al. 1987; Lemieux, Hindsgaul et al. 1988; Spohr and Lemieux 1988; Lemieux, Szweda et al. 1990). Based upon analysis of the binding profiles of lactoseries isomeric structures by BR55-2, we previously postulated that the binding epitope includes the OH-4, and OH-3 groups of the βDGalactose unit, the 6-CH3 groups of the two fucose units, and the N-acetyl group of the subterminal β-D-N-acetyl-glucosamine (βDGlcNAc) (Blaszczyk-Thurin, Thurin et al. 1987). A similar interaction pattern for the involvement of OH-3 and/or OH-4 of the terminal galactose as key polar groups is postulated for the interaction of six other MAbs (Le^a, B blood group, Le^b and I Ma specific antibodies) (Lemieux, Wong et al. 1984; Lemieux, Venot et al. 1985; Spohr, Morishima et al. 1985; Lemieux, Hindsgaul et al. 1988) and lectins (*Ulex europaeus* (Hindsgaul, Khare et al. 1985) and GS4 (Spohr, Hindsgaul et al. 1985)) (Table 1).

Our interpretation of the reactivity patterns (Table 1) indicates that, OH-4^b, OH-3^b, OH-4^c and OH-3^c of the Y molecule might be involved in BR55-2 binding (Figure 1b). A similar hydroxyl group cluster which consists of OH-3^b, OH-4^b and OH-4^c was found to be a part of a topography recognized on Le^b and Y determinants by GS4 (Spohr, Hindsgaul et al. 1985) and on the H-2 structure recognized by *Ulex europaeus* lectin I (Hindsgaul, Khare et al. 1985). Adjacent to the hydroxyl clusters, a lipophilic surface formed by $CH3^c$ and $CH3^d$ as well as O-5^d is postulated to interact with both BR55-2 and GS4. The OH-4^d unit might also contribute to the amphiphilic binding surface since it is present at the edge of the proposed epitope for both BR55-2 and GS4. The involvement of the OH-2 group in α-2-fucose recognition of Le^b with MAb and GS4 is also observed. Since GS4 binds to the common surface in both Le^b and Y, this functional group might also be involved in Y recognition by BR55-2.

The acetamido group of the βDGlcNAc residue, part of the binding epitope for Y of BR55-2, is also involved in binding of specific MAbs with Ma I (Lemieux, Wong et al. 1984) and Le^b (Spohr, Morishima et al. 1985). The specific recognition of the H-2 structure by the lectin *Ulex europaeus* does not significantly involve the N-acetamido group, but does involve the 6-hydroxyl group in the binding reaction (Hindsgaul, Khare et al. 1985). On the other hand, MAbs specific for the type 1 Le^a determinant, recognize both sides of the β-D-N-acetylglucosamine unit with the recognition of CH_2OH being polar in character and that on the acetamido side, nonpolar (Lemieux, Hindsgaul et al.).

Sequence and structural similarities between anti-Y antibodies

To better define the functional groups involved in molecular recognition of Y determinants, we compared the sequence/structural

properties of BR55-2 with several other described anti-Y antibodies, B3 (Brinkmann, Pai et al. 1991), H18A (Kaneko, Iba et al. 1993) and BR96 (Bajorath 1994). The nucleotide sequence and the translated amino acid sequence of the light chain and heavy chain variable domain of BR55-2 is shown in Figure 2. Sequence analysis of BR55-2 shows that VH and V kappa genes are in the VH 7183 family and V kappa C1 family, respectively. Sequence alignment (Figure 3) indicate that the anti-Y antibodies are homologous with each other, with the majority of the residue differences appearing in their heavy chain hypervariable or CDR regions.

Sequence comparison with known immunoglobulin crystal structures (Bernstein, Koetzle et al. 1977) provide a template for the variable regions of the antibodies (Figure 3). The primary structure of the light chain of the anti - cholera toxin antibody TE33 (1TET) and the autoantibody BV04-01 (1CBV) displayed 96% and 89% identity respectively with the BR55-2 light chain (Figure 3). Both of these antibodies have been elucidated by x-ray crystallography as a complex with their respective ligands. Superposition of these two light chains indicate that their CDR conformations are nearly the same except for CDR1 around the sequence tract "S-N-G" of BV04-01. The conformational difference appears to be due to an induced conformation in TE33 upon the binding of the cholera toxin peptide to TE33. Subsequently these structures appear to reflect conformational changes that might be evident upon ligand binding. In the modeling of the antibodies we utilized the TE33 template for the light chain, to reflect these differences even though the antibodies display more sequence identity with BV04-01 within the CDR1 region.

For the heavy chain of BR55-2, we identified two Ig templates, 17/9 (1HIM) and B13i2 (2IGF) which are co-complexed with peptides, displaying 78% and 69% identity respectively with BR55-2 up to CDR3. The antibody 17/9 is complexed with a peptide derived from Influenza Hemagglutinin and B13i2 is complexed with a peptide from Myohemerythrin. Superpositioning of these structures indicate that they display nearly the same conformations up to the CDR3 region (residues 1-95) with an RMS of 0.47 Å, suggesting CDR1 and CDR2 conformations have undergone similar conformational transitions if any. This aspect is of relevance in considering that the CDR2 of 17/9 and BR55-2 each display three glycines (Figure 3) which in principle can adopt unusual phi/psi angles. In comparison with B13i2 which displays a "G-G-S" sequence for residues 53-55, the canonical conformations are equivalent for 17/9 and B13i2.

To model the CDR3 region of the BR55-2 heavy chain, we first examined CDR3 folding patterns of several crystallographicaly known immunoglobulins. Analysis of Ig crystal structures indicate that CDR3 loop regions can adopt conformations that fall into two general classes which have been referred to as kinked and unkinked (Mas, Smith et al. 1992) despite their considerable variability in length, sequence and conformation. Conformational search procedures applied to CDR3 clearly suggest that these are the only conformations available to the base of the loop (Mas, Smith et al. 1992). It has been suggested that packing of residues at the loop base can be used to differentiate between these structures (Mas, Smith et al. 1992).

Based upon the sequence similarities found in B13i2 with the anti-Y antibodies, we utilized this kinked base geometry template and searched for additional loops in the database (Table 2). In splicing each loop of

seven residues identified from the search into the B13i2 template (Table 2), and heating the system to 600 K° and then cooling to 300 K°, we found that the CDR3 loops approached the conformation of the B13i2 loop. In each of the starting conformations the replaced Gly residue at position 5 in the putative loop did not adopt the conformation analogous to the proline in the B13i2 template (Figure 4). The major difference in the two conformation types was centered on Gly H99 (Figure 4). In effect the substituted Gly at position 5 in the B13i2 template points downward, while the substituted Gly in the second conformer type points upward (Figure 4). We found that the B13i2 loop was energetically favorable on average by 4 Kcal/mole. This finding is probably related to the starting bias in the choice of the B13i2 template. Analysis of VH:VL interfaces indicate that the structure of the CDR3 region and the VL:VH association are interdependent as residues at the C terminal end of CDR3 form part of the VH:VL interface. In our modeling we settled on the B13i2 conformer, since both conformers are probably equally likely.

Docking of Y to BR55-2

Using site point information as a guide, we examined the respective CDR domains for residue types implicated in GS4 binding to the difucosylated lactoseries structures. If a site point was associated with a particular residue implicated in Y binding to GS4, we juxtaposed the corresponding functional group on the Y structure in proximity to the site point. We examined the nature of the interactions between the Y structure and the antibodies, identifying residues within 5 Å of the Y structure. Principal interactions with Y for BR55-2 include: Tyr H33, Tyr H50, Ser H52, Asn H52a, Ser H56, Ser H57, His H58, Lys H64, Tyr H98, Gly H99, Ala H100, His L31, Ser L31a, Asn L31b, Ser L92, His L93, Val L94, Pro L95, Phe

L96. In agreement with analysis of BR96 (Bajorath 1994), these residue positions line the antigen combining binding site of BR55-2, indicating the general similarity of the starting templates for the antibody models. In the placement of Y (Figure 5) we observed a range of possible polar and non-polar arrangements illustrated in Table 3 and in figures 5 a-c. Intermolecular interaction calculations for the structures in Figures 5 a-c were within 5 Kcal/mole of each other.

We identified several variations of a putative binding mode for BR55-2 in which many of the contacts were conserved in GS4 and the other anti-Y antibodies (Table 3). In particular, we observed a potential bifurcated hydrogen bond between Asn L31b and OH-3^b and OH-2^d (Figure 6a and c) which mimics Asn 135 binding to a tetrasaccharide form of Leb in GS4 (Table 3). Differences in the glycosidic angles of Y can change the bifurcated hydrogen bond to a single hydrogen bond with OH-2^d (Fig. 5b). The bifurcated hydrogen bonding by Asp 89 in GS4 to OH-3^b and OH-4^b (Table 3) is potentially mimicked by Ser L31a in the antibodies (Figure 6a-c). In B3 we observe a Trp residue contacting OH-3^d (Figure 6a), again mimicking that observed for Le^b binding in GS4 (Table3), while in BR55-2 this contact involves a Tyr. In BR55-2, B3 and H18A Tyr H33 can bind to OH-4^d (Figure 6a-c). Again, variations in the Y structure can introduce a hydrogen bond between Ser L31a and OH-2^d in BR55-2 (Figure 6 a-b). This residue is conserved in B3 and mutated to a Thr in H18A (Figure 3). In BR96 this position is mutated to an Asn (Figure 3), which presumably can still form a hydrogen bond with OH-2^d.

An electrostatic interaction involving His L93 (assuming a physiological pH of 7.0) and the backbone NH group of Val L94 potentially stabilizes the polar interactions with the available hydroxyls of cFuc.

This interaction can approach hydrogen bonding distances (Figure 6 a and b). Val L94 can also interact with cFuc via hydrophobic interactions (Figure 6c) As expected from experimental data (Blaszczyk-Thurin, Thurin et al. 1987), we observe interactions with the GlcNac residue. Our placement of Y results in a hydrogen bond between Ser H56 in BR55-2 with OH-6^{a} (Figure 6b and 5c). This residue is an Ile in H18A, providing a hydrophobic interaction with GlcNAc (Figure 6a). The acetamido group of GlcNAc is involved in either electrostatic or hydrophobic interactions with residue position H58 in the antibodies. Interactions with either the N2 or O7 is dependent on the final conformation of the acetamido group of the GlcNAc residue (Figures 5b and c).

Comparison of binding mode and non-binding mode conformations

We compared the dihedral angles of the Y tetrasaccahride with those of Le^{b} as found in complex with GS4 and with unbound forms established by computational or NMR approaches (Table 4). Molecular dynamics calculations (Table 4a columns 1 and 2) of unbound Le^{b} structures indicate that low energy conformers fluctuate about 10 degrees and are within 3-4 Kcal/mol around a single minimum energy conformation represented by NMR results (Table 4a column 3), HSEA (Table 4b column 2) calculations and with the Le^{b} structure observed in GS4 (Table 4a column 4 and Table 4b column 1). A converged structure for Le^{b} (Table 4a column 5 and Table 4b column 3), which started from an average of low energy conformers identified from a grid search, was found to be within this range of angle fluctuations.

To determine the extent to which final minimized models are dependent on the starting conformation, we used the HSEA conformer (Table 4b column 2) as a starting geometry. Minimization of this

geometry resulted in the same conformer (Table 4a column 5 and Table 4b column 3) as that starting from the binding mode conformation of Le^b in GS4 (Table 4a column 4, Table 4b column 1). The HSEA derived starting conformation in Table 4b column 2, was found to be only 0.8 Kcal/mole above the converged conformer (Table 4a column 5 and Table 4b column 3), with the Le^b binding mode conformation 2.8 Kcal/mole above the converged structure. These calculations confirm that the binding mode of Le^b for GS4 and the HSEA derived structure are local minimas within the range of fluctuations for the glycosidic angles observed from MD calculations (Mukhopadhyay and Bush 1991).

Performing similar calculations for the Y structure it was found that averaged low energy forms identified from a grid search, as well as the HSEA starting conformation, led to a converged conformer (Table 5 column 5) similar to that found for the Le^b structure (Table 4a column 3) for the representative units. Minimization of the three binding mode conformations (Table 5 columns 1-3) led to different local minimas involving the Fucα(1->2) moiety. The conformer in Table 5 column 3 led to the converged structure observed in the grid search (Table 5 column 5), with the conformers in column 1 and 2 converging to that in column 6. The two binding mode structures in Table 5 columns 1 and 2, were 2.7 Kcal above their energy optimized structures, with that in column 3, being 1.5 Kcal/mole above its optimized structure. In comparison, the HSEA conformation (Table 5 column 4) was 2.3 Kcal above its optimized structure shown in column 5. We found that the converged structures shown in Table 5 columns 5 and 6 were only 0.1 kcal/mole different in energy, suggesting that the range of conformations described for the binding modes are equally probable. Fitting the Le^b structure to these Y

binding mode geometries indicate however that they are 8 Kcal/mole and above the Y binding mode structures. This suggests that the Le^b structure might not be energetically favorable to adopt the Y binding modes for BR55-2.

To further evaluate the ability of the Le^b structure to populate the Y binding mode for BR55-2, we examined the glycosidic angle distributions of 100 energy minimized structures derived from an MD calculation. The starting conformation for both Y and Le^b structures was that in Table 5 column 3. Figure 6 summarizes these results in which we observe that the Fucα(1->2) glycosidic angles in Le^b are more restricted than found for the Y structure. For the Le^b structure φ^1 is restricted within 3 degrees of the crystal structure angle, while ψ^1 is restricted within 1 degree. For the Y structure, φ^1 and ψ^1 display both positive and negative angle ranges, reflective of the optimized structures in Table 5, columns 5 and 6. Repeating this calculation, using a dielectric of 1, we observed that N2 of the acetamido group of the GlcNAc residue in the Le^b structure forms a hydrogen bond with O5 of dFuc, restricting φ^1 to negative angle ranges. This restriction results in higher enegry forms (Mukhopadhyay and Bush 1991). Changing the dielectric for the Y structure has little effect on the distribution of angle range, but does affect the relative conformation population within the angle range.

Discussion

Analysis of the spatial orientation of contact residues on antibodies has been successfully used to identify topologic relationships between antibodies and ligand receptors (Williams, Kieber-Emmons et al. 1991; Lin, Kieber-Emmons et al. 1994). In our previous studies (Williams, Kieber-Emmons et al. 1991), a crystal structure of an analogous

carbohydrate-protein complex was useful in describing potential binding mode conformations for sialylic acid binding to a surrogate antibody. In the present studies a lectin (GS4) crystal structure and mapping information (Table 1), was employed to elucidate molecular recognition properties of lectin surrogate antibodies that bind to the Y determinant. A conservative approach in placing the Y tetrasaccharide structure was taken in which functional group contacts are conserved on the Le^b/Y structure as observed in GS4. The restraints imposed based on this criteria provided a model consistent with that observed for GS4 binding to the lactoseries structure.

The models of the antibodies indicate that they are representative of the groove type architecture suggested some time ago for anti-carbohydrate antibodies (for review of carbohydrate recognition by antibodies see (Cygler 1994)). Current procedures for predicting ligand - antibody interactions is limited, mainly due to the conformational flexibility of ligands and the role of solvent in mediating ligand recognition and binding. However, modeling does provide an avenue for hypothesis testing when relevant alternative structural or binding information is available. In the present case, structures from lactoseries reactive antibodies and the crystal structure of GS4 have defined potential sites on Lewis structures that dictate their specificity (Table 1).

Comparison of calculated and NMR derived conformers of precursors of type 1 (Galβ1$\rightarrow$3GlcNAc) and type 2 (Galβ1$\rightarrow$4GlcNAc) chains show that they display very similar topographical features (Fig 1a). Spohr et al (Spohr, Hindsgaul et al. 1985) have shown that the binding of these structures by GS4 should occur at the termini of the molecule involving

the common surface to both structures based on a wide number of chemically modified structures related to Le^b and Y human blood group determinants. The modeled structures for Y binding to the antibodies appear to reflect salient features for binding of Le^b to GS4 and is consistent with antibody reactivity profiles to the lactoseries analogues (Table 1). Important functional group contacts observed in GS4 are largely conserved in the antibodies (Table 3), however, some of the interactions differ among the residues and may define their overall specificity. All the sugar moieties (aGlcNac, bGal, cFuc, and dFuc) are stabilized by hydrogen bond and hydrophobic interactions (Table 3). The residues that stabilize cFuc, dFuc and bGal are conserved in the BR55-2 and B3 prototypes. Residues Ser31La Asn31Lb, stabilize both bGal, and dFuc moieties through bifurcated hydrogen bonds. The cFuc moiety is stabilized by His 93L and the backbone NH group of Val 94L. For the fucose moieties, mimicry of GS4 binding is clearly evident in the role played by Asn L31b and Trp H98 of B3, in that these residues are both preserved in GS4 in contacting the respective functional groups.

Based on the oligosaccharide specificity, lectins and antibodies that distinguish between the difucosylated type 1 and 2 lactoseries structures, it appears that a critical recognition feature for type 1 and 2 chain specificity is an involvement of the 6-CH_2OH and/or acetamido group of GlcNAc residue in the antibody combining site. The involvement of the β-D-N-acetylglucosamine residue in the binding epitope for Y specific MAbs was previously postulated (Blaszczyk-Thurin, Thurin et al. 1987). In BR55-2 model, the 6-CH_2OH of GlcNAc interacts with a Ser residue (position 56H) in BR55-2. In H18A, this hydrogen bond is replaced by hydrophobic interactions with Ile 56. We also find that the N-acetamido

group forms both polar and nonpolar interactions. The acetamido group in GlcNAca is neutralized by His 58 in BR55 and by Ala in B3 and Tyr in H18A. These differences probably translates into different affinities for Y for these antibodies. In the crystal structure of GS4, GlcNAc is completely exposed and not involved in any interactions. Thus, the GlcNAc moiety is postulated to play a key role in the recognition of Y by the antibodies, while retaining interactions which are shared with GS4. Subsequently, the interaction of the βDGlcNAc residue with Y specific antibodies is a driving mediator in their specificity.

Structural studies on Lewis antigens have generally substantiated that conformations are determined mainly by steric repulsion brought about by changes in the glycosidic dihedral angles. Molecular dynamics calculations on Lewis antigen structure prototypes indicate the lack of spontaneous conformational transitions to other minima during the simulations, suggesting that these oligosaccharides maintain well-defined conformations with relatively long lifetimes (Mukhopadhyay and Bush 1991). These results further indicate that hard sphere or rigid-geometry calculations, albeit in the absence of solvent, provides a good picture of the steric repulsions that modulate the conformational properties of the Lewis antigens.

Our minimization's, starting from binding mode geometry's, were found to converge to such conformations. Subsequently, the binding mode conformation described in Figure 6c and in Table 5 column 3, are in excellent agreement with expectations for the Y structure derived from consideration of both MD (Mukhopadhyay and Bush 1991) and NMR (Cagas and Bush 1990; Cagas and Bush 1992) studies. This binding mode is stabilize by BR55-2 by 5 kcal/mole in intermolecular energy calculations.

However, it appears that the Y structure might also fluctuate about a binding mode conformation (Table 4c columns 1 and 2 and Figure 6 a and b) that deviates from the well-defined conformation represented in Table 5 column 3. The principle deviation lies in the dFuc(1->2)bGal glycosidic torsion angles. In our minimization's for the Lewis Y structure we find that the energy difference between the converged local minima derived from the antibody binding modes is only 0.1 Kcal/mole. In contrast, we found that analogous Le^b structures are 8 Kcal/mole and above these low energy conformers which are typically not observed from previously reported MD calculations (Mukhopadhyay and Bush 1991) and our own calculations.

To further evaluate the binding mode conformers, we used the program Search and Compare to explore the interrelationship between the glycosidic angles and their mutual ability to populate their respective binding mode conformations. For the Lewis Y structure, we observed a very large range of conformers (2000) within 2.5 Kcal that include the HSEA and NMR values (1.4 Kcal above the lowest energy conformer). Subsequently, there is a very large population of structures relative to the identified binding mode phi/psi values. For Lewis b, we observed a wide range of conformers within 2 Kcal/mole for the bGal and cFuc units including values for the Y binding mode. However, only very high energy conformers (7-200 Kcal/mole) were observed for the dFuc moiety relative to the range of values representative of the dFuc residue of Y. Steric repulsion involving the methyl group of the dFuc moiety restricts its conformational flexibility in the Le^b structure. Subsequently, while residue contacts might be shared between the anti-Y antibodies and GS4 for either type 1 or type 2 difucosylated structures, only the Y structure

might exhibit fluctuation about the dFuc(1->2)bGal glycosidic linkage angles or adopt a binding mode conformation that is intrinsically more stable than for Le^b. These studies indicate that binding mode conformations required for antibody recognition of key polar groups on the Y structure might not be shared for the difucosylated Lewis structures, which has hitherto been under appreciated. These latter conclusions reached in the present studies await confirmation with the advent of a refined structure for the anti-Y antibody B96 in complex with a Y epitope (Chang, Jeffrey et al. 1994) and our own studies on the crystallization of BR55-2.

The interplay between carbohydrates and proteins is of fundamental importance in a number of different biological processes. In particular, cell adhesion and cell recognition events are often mediated by protein-carbohydrate interactions forming a basis in the etiology of tumors. The inhibition of these interactions is a possible point of therapeutic intervention. An understanding of the 3-dimensional basis for the molecular recognition of Y by these antibodies can be applied for future diagnosis in tumor progression and micrometastasis as well as active immunotherapy. For example, the model of BR55-2 emphasizes the role played by the fucose methyl groups facing the floor of the antibody combining site. These methyl groups might be mimicked by methyl group containing amino acid residues in a designed peptide or anti-idiotypes that compete with Y for anti-Y antibodies. A peptide that mimics the Y antigen has been described (Hoess, Brinkmann et al. 1993) that contains such methyl groups.

The use of peptides or anti-idiotypes that mimic carbohydrate epitopes might also serve as a unique vaccine strategy for induction of anti-tumor immune responses. Peptides that mimic carbohydrates have

shown to be capable of inducing anti-carbohydrate immune responses (Westerink, Giardina et al. 1995). A peptide or engineered antibody mimic that expresses three-dimensional shapes resembling structural epitopes of the Lewis Y carbohydrate antigen might be recognized as a foreign molecule, breaking any tolerance to human self-antigens (Kieber-Emmons, Ward et al. 1986; Hellstrom and Hellstrom 1989; Hellstrom and Hellstrom 1992; Hu, Hellstrom et al. 1992). Further structural studies of Y antigen forms binding to anti-Y antibodies will provide information relevant to vaccine design strategies and improved immunotherapeutics.

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Figure Legends

Figure 1 Similarity in structure and recognition patterns observed for Lewis Y and Leb structures. a. Superposition of Y (dark light) and Le^b (light line) structures highlighting the N-acetyl and hydroxymethyl groups projected on opposite sides of the type 1 and 2 difucosylated structures. Superpositioning indicates that inspite of the change of glycosidic linkage from β1-3 to β1-4 in the type 1 and 2 chains, resulting conformational features of the respective sugar moieties are still shared forming a common topography. b. Solid sphere rendering of the Y tetrasaccharide core, implicating the surface recognized by BR55-2 and lectins by considering mapping information in Table 1. The a,b,c,d designation refer to atoms on the βDGlcNac, βDGal, αLFuc(1→3) and αLFuc(1→2) units, respectively. Interacting hydroxyl groups are shown in red and labeled accordingly. The Fucose methyl groups involved in hydrophobic interactions are shown in magenta. The acetamido group of GlcNAc is colored yellow. The arrangement of interacting atoms form a continuos surface on the face of the core.

Figure 2. Nucleic acid and amino acid sequence of the light chain (A) and heavy chain (B) of BR55-2. The first amino acid of the mature protein is double underlined. Complementarity determining regions (CDRs) are also underlined.

Figure 3. Sequence alignment of the light (a) and heavy chains (b) of BR55-2, B3, H18A and BR96, among themselves and with template crystal structures as identified by Blast search of the protein crystallographic

database on the NCBI Blast server. Dashes indicate identities with respect to BR55-2. Numbering corresponds to that of Kabat (Kabat, Wu et al. 1987). Only a partial sequence has been presented for BR96 (Bajorath 1994). The structure of the light chain of TE33 (Brookhaven code 1TET (Bernstein, Koetzle et al. 1977)) was used as a template for the modeling of BR55-2 and B3 light chain. For the heavy chain the B13i2 structure (2IGF) was used as the template. This structure displayed equivalent length CDRs with respect to the anti-Y antibodies. The antibody 17/9 has a longer CDR3 region than the anti-Y antibodies.

Figure 4. Stereoview of BR55-2 looking down the binding site emphasizing the sequence dependent transition of the CDR3H loop. The difference between the structures is the position of the Gly as indicated (see text). Figure was drawn using MOLSCRIPT (Kraulis 1991)

Figure 5. Stereoview of BR55 complexed with Y. Both BR55-2 and B3 are representative of the groove-like architecture. The detailed interactions are described in Figure 6 and text. Figure was drawn using MOLSCRIPT (Kraulis 1991)

Figure 6. Summary of hydrogen bonding and hydrophobic interactions identified for Y binding to BR55-2. Variations of a putative binding conformation which emphasizes the shared topography between Le^b and Y are shown with respect to BR55-2. Residue substitutions derived from B3 or H18A are highlighted. Interaction energies for the Y structure for the antibodies range from -59 (figure a) to -64 (Figure c) kcal/mole. Hydrogen bonding distances identified by the program LIGPLOT (Wallace,

Laskowski et al. 1994) reflect idealized heavy atom distances less than 3.3 Å. The overall interactions of BR55-2 and the other antibodies with Y are very similar. In a-c, the residues that stabilize cFuc, dFuc and bGal are conserved in the antibodies. Residues Asn L31b, Ser L31a potentially stabilize both bGal, dFuc moieties through bifurcated hydrogen bonds. These residue are mutated to Thr in H18A and an Asn in BR96. The cFuc moiety is potentially stabilized by His93 and Val94 of light chain. The acetamido group in aGlcNac is neutralized by His58 in BR55-2. Ser H56 forms a hydrogen bond with OH-6 of aGlcNAc. On the other hand, in H18A this hydrogen bond is replaced by hydrophobic interactions with Ile 56H. In the crystal structure of GS4 aGlcNAc is completely exposed and not involved in any interactions. Thus, aGlcNac plays a key role in the recognition while BR55-2 interacts with the common topography of Le^b and Y.

Figure 6. dFuc glycosidic angle distribution of Lewis tetrasaccahride structures. a. φ^1,ψ^1 distribution for the Y structure. b. φ^1,ψ^1 distribution for the Le^b structure. Structures were generated using a dielectric of 80 to reduce electrostatic effects on final conformations.

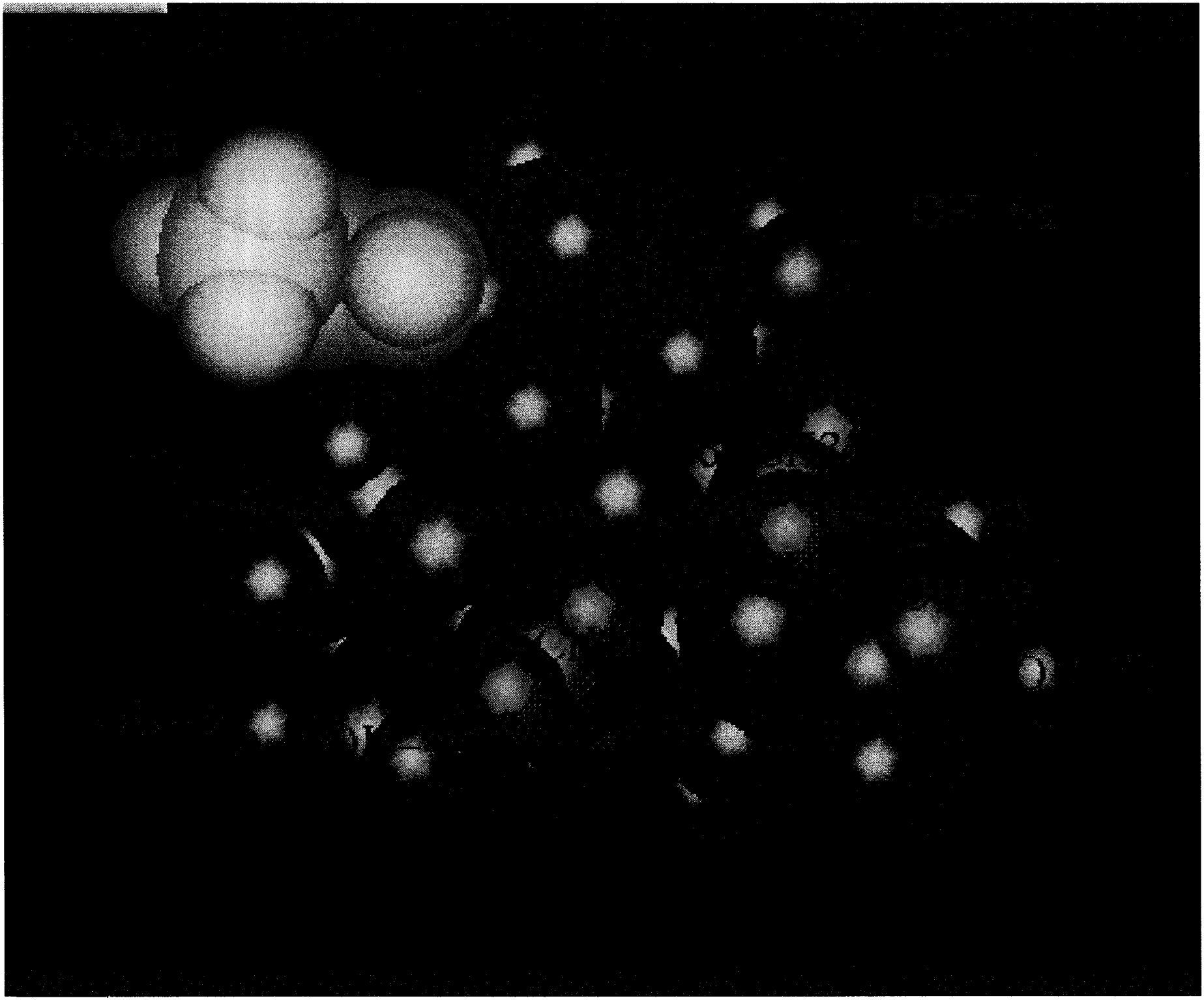


Fig 1b

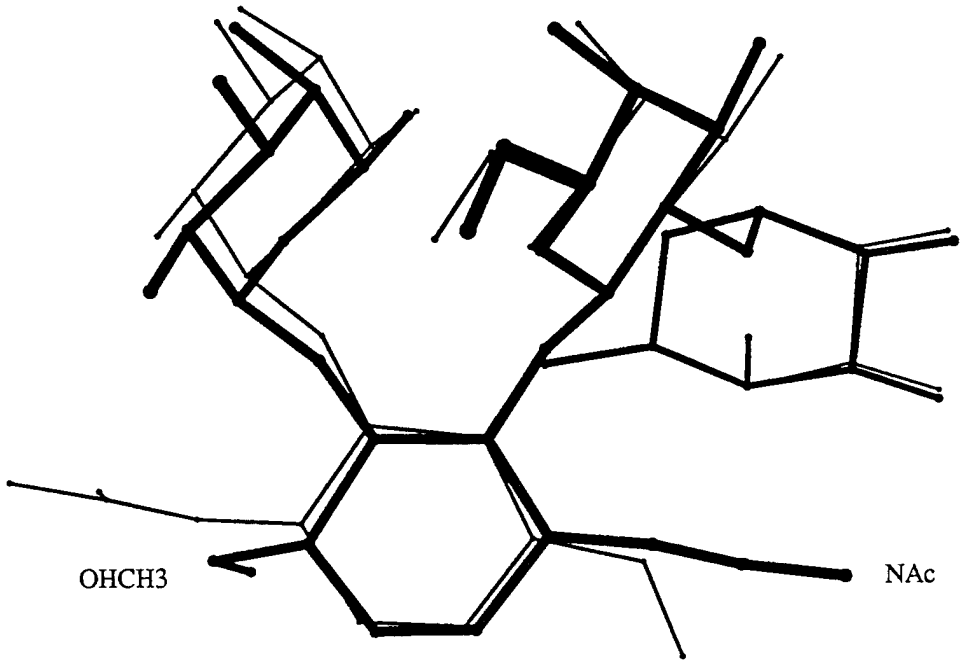


Fig 1a

```
        10         20         30         40         50         60
ATGAACTTGGGGCTCAGCTTGATTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTGTGAA
 M  N  L  G  L  S  L  I  F  L  V  L  V  L  K  G  V  Q  C  E

        70         80         90        100        110        120
GTGAAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCTGAAACTCTCC
 V  K  L  V  E  S  G  G  G  L  V  Q  P  G  G  S  L  K  L  S

       130        140        150        160        170        180
TGTGCAACCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACTCCA
 C  A  T  S  G  F  T  F  S  D  Y  Y  M  Y  W  V  R  Q  T  P

       190        200        210        220        230        240
GAGAAGAGGCTGGAGTGGGTCGCATACATTAGTAATGGTGGTGGTAGTAGCCATTATGTA
 E  K  R  L  E  W  V  A  Y  I  S  N  G  G  G  S  S  H  Y  V

       250        260        270        280        290        300
GACAGTGTAAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTACCTG
 D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  T  L  Y  L

       310        320        330        340        350        360
CAAATGAGCCGTCTGAGGTCTGAGGACACAGCCATGTATCACTGCGCAAGGGGGATGGAT
 Q  M  S  R  L  R  S  E  D  T  A  M  Y  H  C  A  R  G  M  D

       370        380        390        400        410
TACGGGGCCTGGTTTGCTTACTGGGGCCAGGGGACTCTGGTCACTGTCTCTGCA
 Y  G  A  W  F  A  Y  W  G  Q  G  T  L  V  T  V  S  A
```

Figure 2 A

```
        10         20         30         40         50         60
ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGCAGTGAT
 M  K  L  P  V  R  L  L  V  L  M  F  W  I  P  A  S  S  S  D

        70         80         90        100        110        120
GTTTTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATC
 V  L  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S  I

       130        140        150        160        170        180
TCTTGCAGATCTAGTCAGAGCATTGTACATAGTAATGGAAACACCTATTTAGAATGGTAC
 S  C  R  S  S  Q  S  I  V  H  S  N  G  N  T  Y  L  E  W  Y

       190        200        210        220        230        240
CTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTCCAAAGTTTCCAACCGATTTTCT
 L  Q  K  P  G  Q  S  P  K  L  L  I  S  K  V  S  N  R  F  S

       250        260        270        280        290        300
GGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGC
 G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K  I  S

       310        320        330        340        350        360
AGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGCTTTCAAGGTTCACATGTTCCATTC
 R  V  E  A  E  D  L  G  V  Y  Y  C  F  Q  G  S  H  V  P  F

       370        380        390
ACGTTCGGCTCGGGGACAAAGTTGGAAATAAAA
 T  F  G  S  G  T  K  L  E  I  K
```

Figure 2 B

Light Chain

```
                                  10                  20                  31 a b c d e 32
BR55 D V L M T Q T P L S L P V S L G D Q A S I S C R S S Q S I V H S N G N T Y L E W
B3   - - - - - - S - - - - - - - - - - - - - - - - - - - I - - - - - - - - - - - - -
H18A - - - - - - - - - - - - - - - - - - - - - - G - G - - - I - T - - - - - - - - -
BR96   -                                                   - - - I - - - N - - - - - -
TE33 - - - - - - - - - - - - - - - - - - - - - - K - - - - - - - - S - - - - F - -
BV04 - - V - - - - - - - - - - - - - - - - - - - - - - - - - - L - - - - - - - - - H -

                  40                  50                  60                  70
BR55   Y L Q K P G Q S P K L L I S K V S N R F S G V P D R F S G S G S G T D F T L K I S R
H184   - - - - - - - - - - - - - - Y - - - - - - - - - - - - - - - - - - - - - - - - - - -
B3     - - - - - - - - - - - - - - Y - - - - - - - - - - - - - - - - - - - - - - - - - - -
BR96                           L   - - -                   -                   -
TE33   - - - - - - - - - - - - - - Y - - - - - - - - - - - - - - - - - - - - - - - - - - -
BV04   - - - - - - - - - - - - - - Y - - - - - - - - - - - - - - - - - - - - - - - - - - -

                80                  90                  100
BR55   V E A E D L G V Y Y C F Q G S H V P F T F G S G T K L E I K
H184   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
B3     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
BR96                           - - - - - - -
TE33   - - - - - - - - - - - - - - - - I - - - - - - - - - - - -
BV04   - - - - - - - - - F - S - S T - - - L - - - A - - - - - L -
```

Figure 3a

Heavy Chain

	10									20									30									
BR55	E	V	K	L	V	E	S	G	G	G	L	V	Q	P	G	G	S	L	K	L	S	C	A	T	S	G	F	T	F	S	D	Y	Y	M
B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H18A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BR96																										-	-	-	-	-	-	-		-
B13i2	-	-	Q	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	R	C	A	-
Fab17/9	-	-	Q	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	A	-	-	-	S	-	-	S	-	G	-

	40														50	52	a	53						60									
BR55	Y	W	V	R	Q	T	P	E	K	R	L	E	W	V	A	Y	I	S	N	G	G	G	S	S	H	Y	V	D	S	V	K	G	R	F
B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	D	S	-	A	A	-	S	-	T	-	-	-	-	F
H18A	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	D	I	P	Y	-	L	-	T	-	-	-	-	F
BR96																			Q	-	-	D												
B13i2	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	S	-	-	S	Y	T	F	-	P	-	T	-	-	-	-	F
Fab17/9	S	-	-	-	-	-	-	D	-	-	-	-	-	-	-	T	-	-	-	-	-	Y	T	Y	-	P	-	-	-	-	-	-	-	F

	70										80	82	a	b	c	83						90													
BR55			T	I	S	R	D	N	A	K	N	T	L	Y	L	Q	M	S	R	L	R	S	E	D	T	A	M	Y	H	C	A	R	G	M	D	Y
B3	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	I	-	S	-	-	-	-	L	A	W			
H18A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	Y	-	-	-	-	K	Y	-			
BR96			-																											-	L	-	D			
B13i2	I	-	-	-	N	-	-	R	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	T	-	Y	S	S	D			
Fab17/9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	S	-	-	-	-	-									

	100															
BR55	G	A	W	F	A	Y	W	G	Q	G	T	L	V	T	V	S	A
B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S
H18A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BR96	-	-	-	-	-	-											
B13i2	P	F	Y	-	D	-	-	-	-	-	-	T	L	-	-	-	S
Fab17/9																	

FIGURE 3b

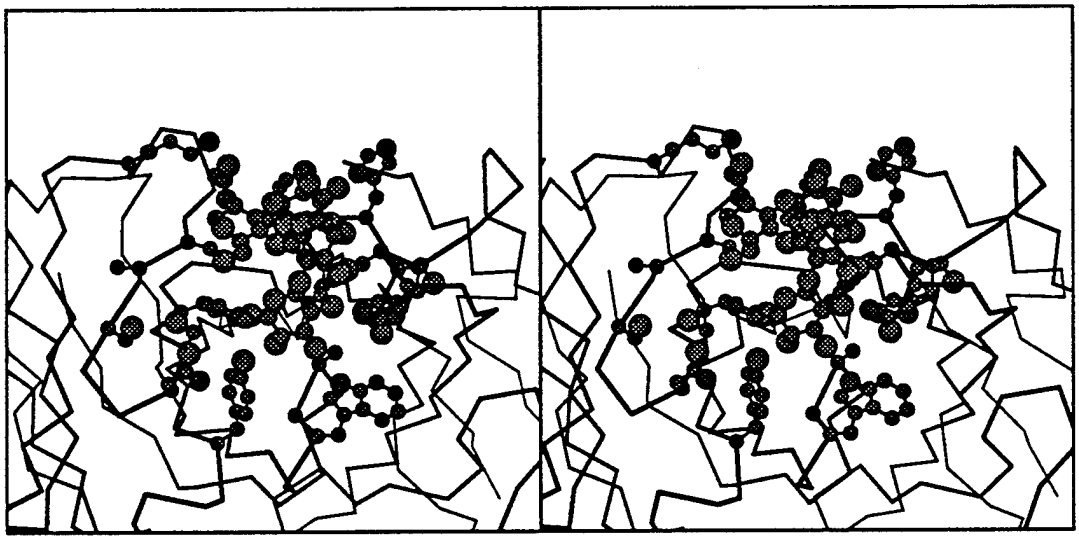
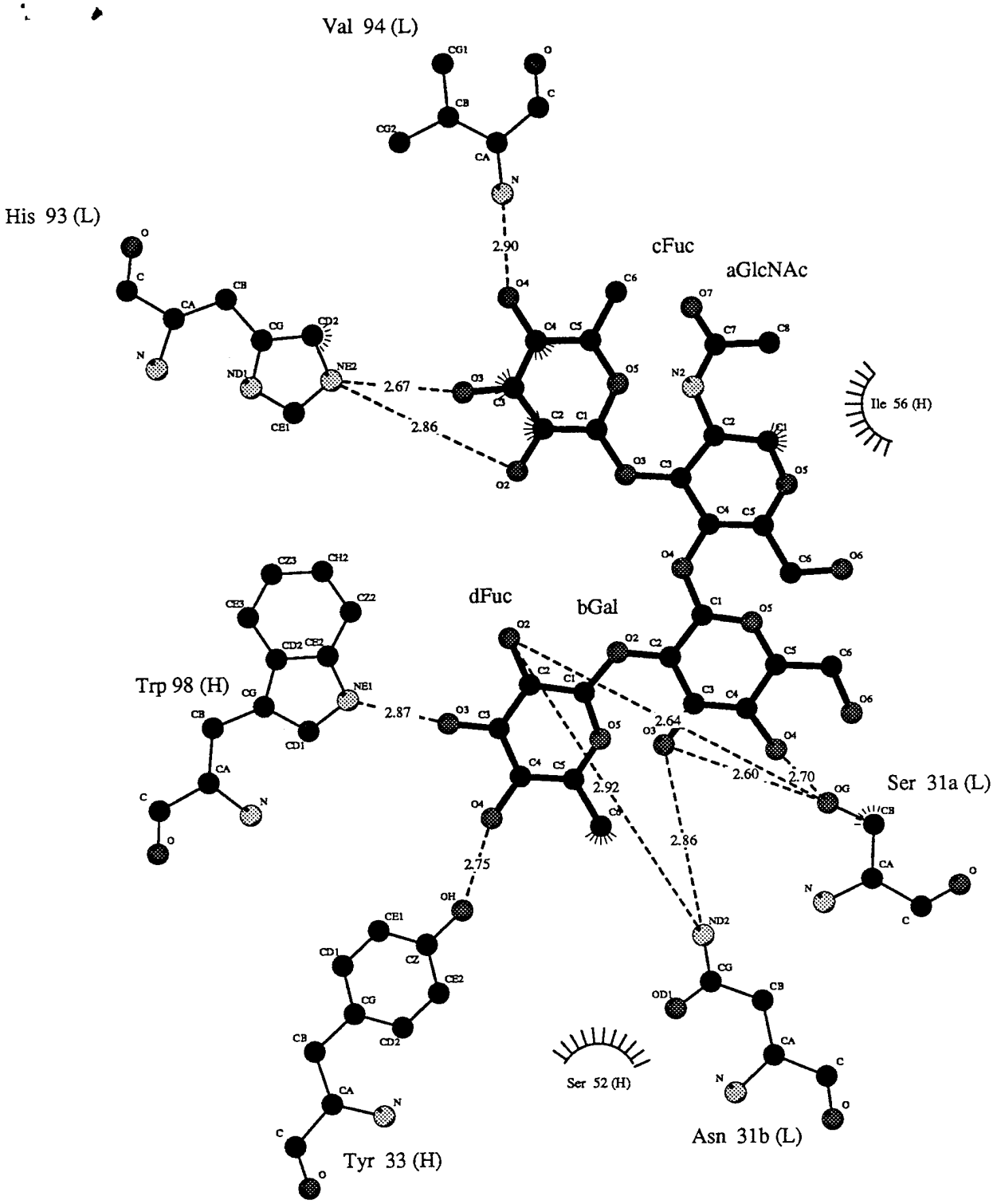


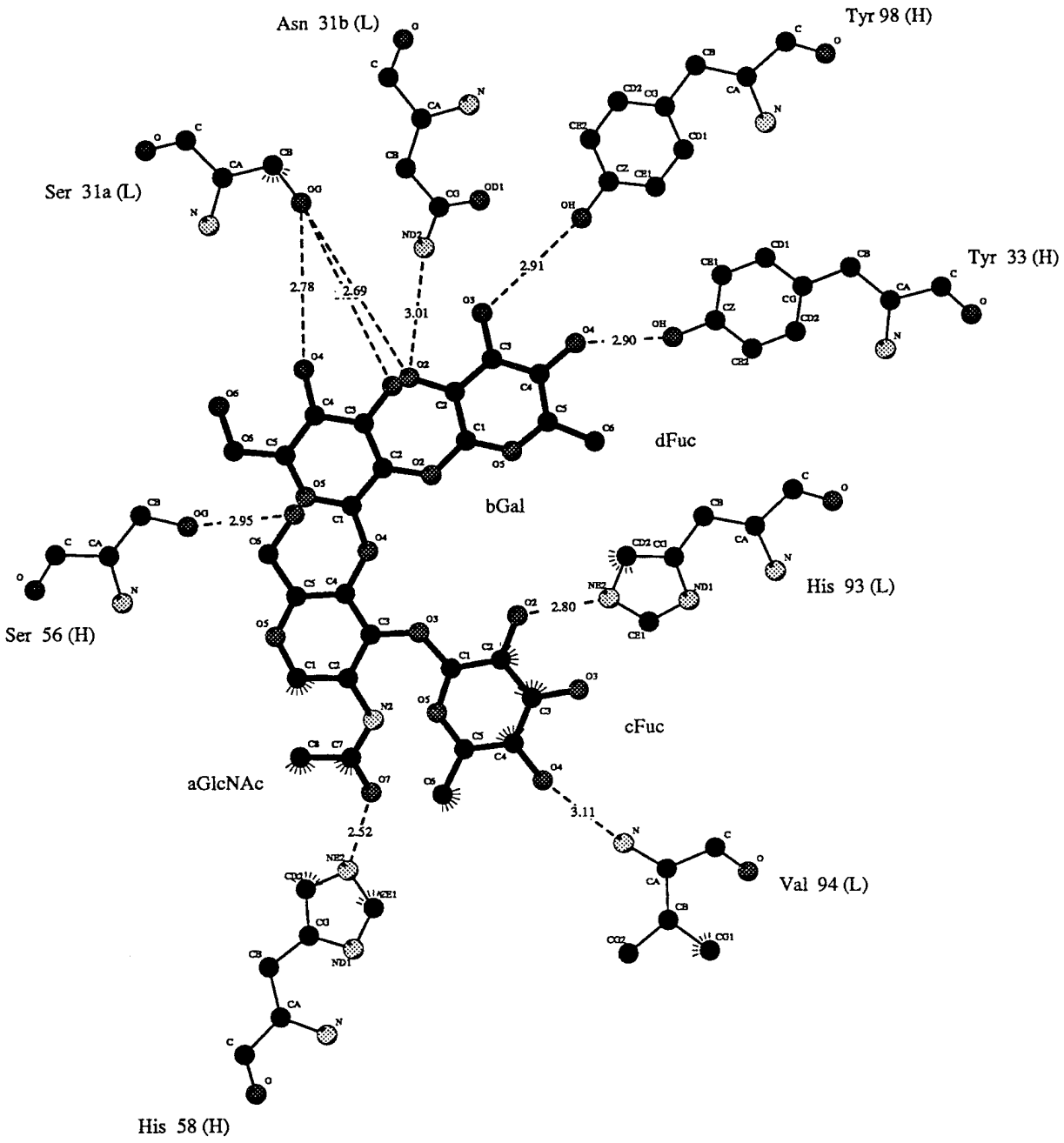
Fig 8



Key

- Ligand bond
- Non-ligand bond
- 3.0 Hydrogen bond and its length
- His 53 Non-ligand residues involved in hydrophobic contact(s)
- Corresponding atoms involved in hydrophobic contact(s)

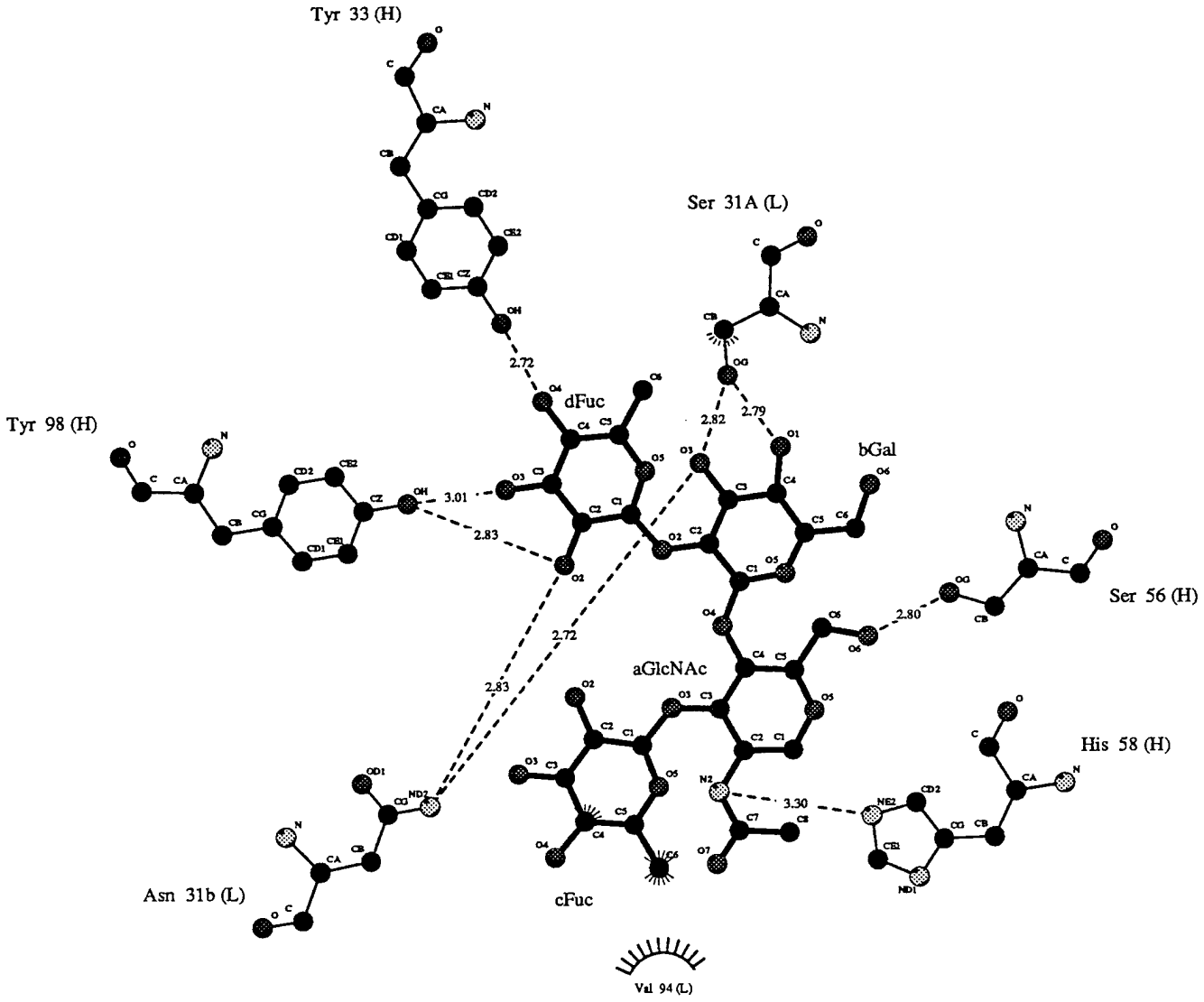
Fig 1c



Key

- Ligand bond
- Non-ligand bond
- 3.0 Hydrogen bond and its length
- His 53 Non-ligand residues involved in hydrophobic contact(s)
- Corresponding atoms involved in hydrophobic contact(s)

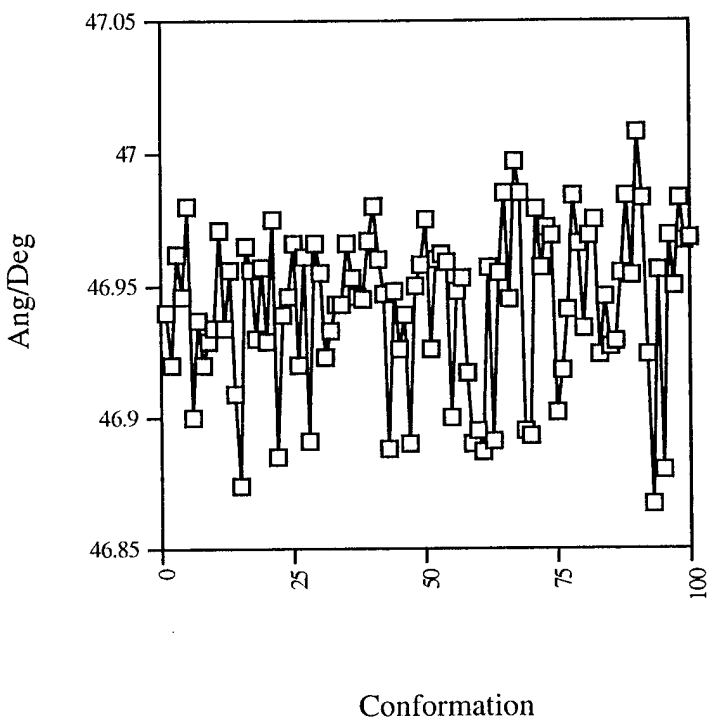
F.J 6b



Key

- Ligand bond
- Non-ligand bond
- 3.0 Hydrogen bond and its length
- His 53 Non-ligand residues involved in hydrophobic contact(s)
- Corresponding atoms involved in hydrophobic contact(s)

Fig 6c


47.05
47
46.95
46.9
46.85
Ang/Deg
0
25
50
75
100
Conformation

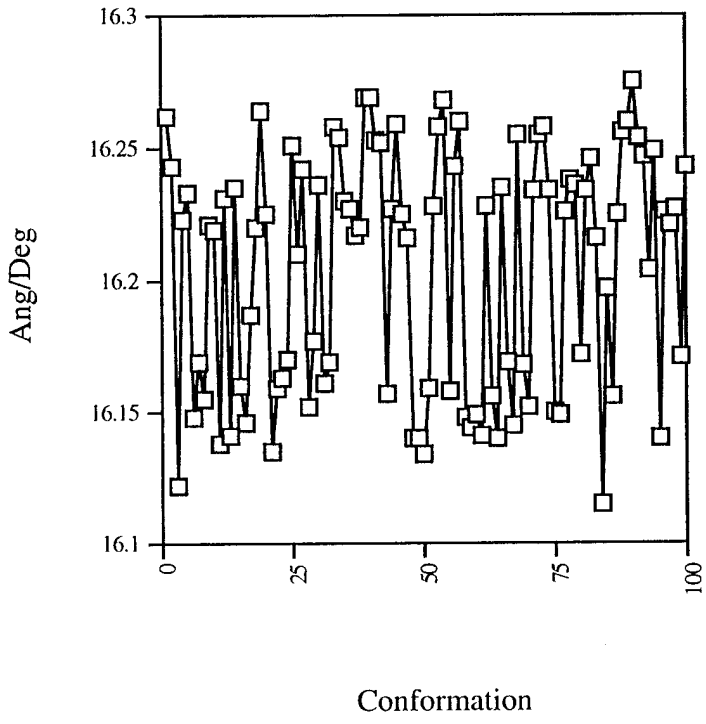

16.3
16.25
16.2
16.15
16.1
Ang/Deg
0
25
50
75
100
Conformation

Figure 7 a.

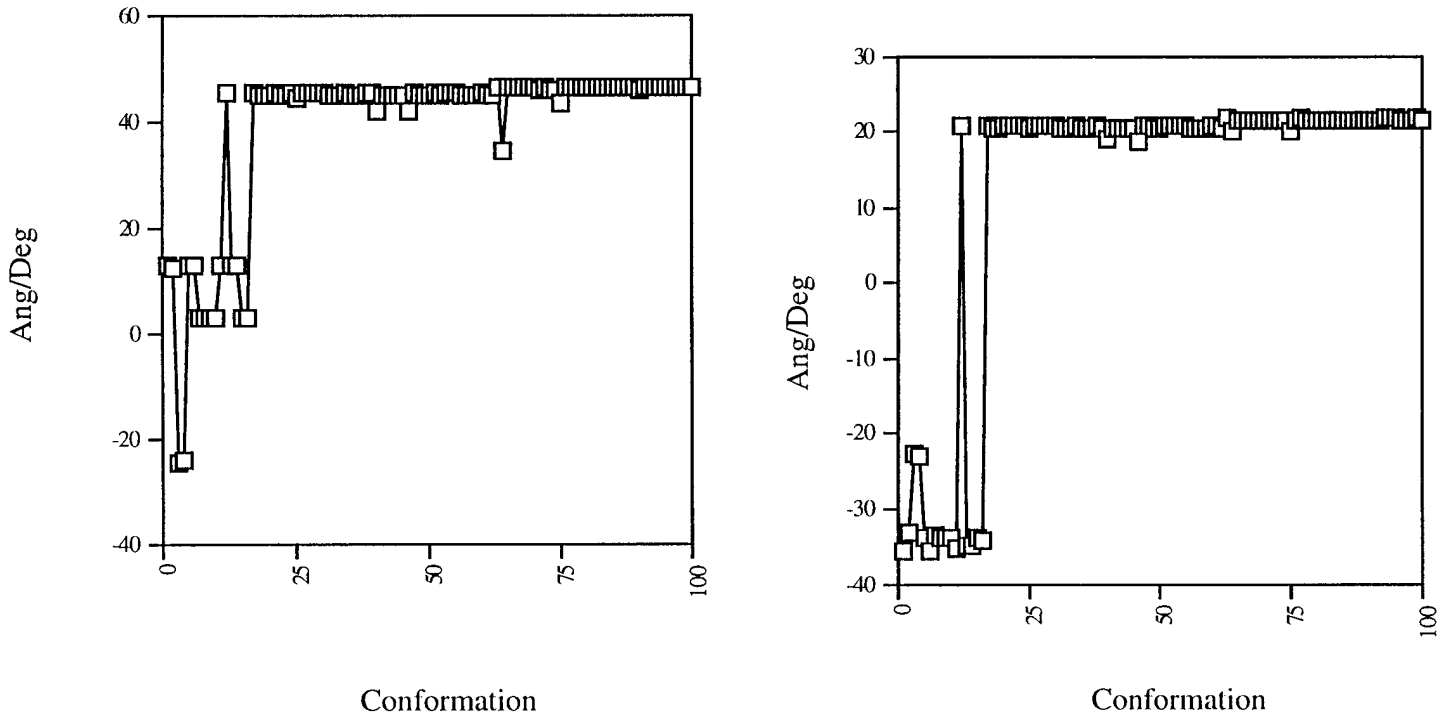

Ang/Deg
60
40
20
0
-20
-40
0
25
50
75
100
Conformation
Ang/Deg
30
20
10
0
-10
-20
-30
-40
0
25
50
75
100
Conformation

Figure 6 b.